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El receptor del factor de Crecimiento Epidérmico (EGFR): Nueva diana terapéutica en la patología renal.

Sandra Rayego Mateos

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Nueva diana terapéutica en la patología renal.

Memoria que presenta la licenciada en biología Sandra Rayego Mateos para optar al grado de Doctor por la Universidad Autónoma de Madrid

Directores de tesis:

Dra. Marta Ruiz Ortega y Dr. Jesús Egido de los Ríos

Instituto de Investigaciones Sanitarias-Fundación Jiménez Díaz,

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RESUMEN

La mayor parte de las enfermedades renales crónicas (ERC) se caracterizan por una inflamación crónica y una fibrosis progresiva, que conduce a una insuficiencia renal terminal. Existen diferentes factores/mediadores que participan en la progresión de la ERC, entre los que destacan CCN2, PTHrP y TWEAK. El EGFR es un receptor de membrana de tipo tirosina quinasa que se expresa en glomérulo y túbulointersticio renal. Estudios previos han descrito el papel clave del EGFR en la progresión de la fibrosis renal. Sin embargo, no existen apenas datos concluyentes sobre su papel en el proceso inflamatorio. El objetivo general de esta tesis es determinar la contribución de la vía del EGFR en el daño renal experimental, investigando sus efectos en los procesos de inflamación y fibrosis, y evaluando los mecanismos intracelulares implicados, con el fin de mejorar las estrategias terapéuticas actuales empleadas en pacientes con ERC. La activación del EGFR se puede producir mediante activación directa o mediante "transactivación", esta última dependiente de MMPs/ADAMs. Los resultados de esta tesis han demostrado que CCN2 es un nuevo ligando del EGFR que se une directamente a este receptor y lo activa, de forma independiente de MMPs/ADAMs. Por otro lado, diversos factores implicados en el daño renal, tales como PTHrP y TWEAK, desencadenan la transactivación del EGFR en el riñón. Los mecanismos implicados en la transactivación del EGFR inducida por PTHrP, vía el receptor acoplado a proteínas G PTH1R, incluyen MMPs, PKC y Src. Por otro lado, TWEAK, mediante su interacción con su receptor Fn14, transactiva el EGFR por activación de ADAM17 y liberación de los ligandos HB-EGF y TGF-α. Estudios experimentales previos sugieren que el bloqueo de la vía del EGFR podría ser una diana antifibrótica en la patología renal. En estudios *in vitro* hemos observado que la activación directa del EGFR por CCN2, y la transactivación de esta ruta por PTHrP es capaz de regular los eventos asociados a la transición epitelio mesenquimal, proceso que contribuye a la fibrogénesis renal. Los resultados obtenidos en esta tesis estudiando los modelos experimentales de daño renal por administración sistémica de CCN2(IV) o de TWEAK en ratón, demuestran que la activación de la vía del EGFR se asocia a un proceso inflamatorio que se previene con el bloqueo farmacológico de esta ruta. El bloqueo de la transactivación del EGFR inducida por TWEAK modulando el eje ADAM17/EGFR, disminuyó la respuesta inflamatoria, sugiriendo que el bloqueo de este eje podría ser una buena opción terapéutica para enfermedades inflamatorias renales. La enfermedad renal crónica se caracteriza por una deficiencia en los niveles de vitamina D. Diversos datos experimentales demuestran que los agonistas del receptor de vitamina D (VDRAs) ejercen efectos antinflamatorios beneficiosos en ERC. En esta tesis hemos observado que el tratamiento con el VDRA Paricalcitol inhibió la transactivación del EGFR inducida por TWEAK así como la activación de mecanismos intracelulares posteriores, incluyendo la sobreexpresión de factores proinflamatorios y la presencia de células infiltrantes en el riñón. El factor de trascripción NF-κB juega un papel clave en la regulación de la respuesta inflamatoria renal. TWEAK es una de las pocas citoquinas que activa la vía canónica y la no canónica de NF-κB. El bloqueo de la trasactivación de EGFR inducido por TWEAK mediante diferentes abordajes farmacológicos, como Paricalcitol, no inhibió la activación de la vía canónica de NF-κB1 mientras que si bloqueó la activación de la vía no canónica NF-κB2 y la inducción de quimioquinas específicas de esta ruta. Estos resultados muestran un nuevo mecanismo antinflamatorio de los VDRAs mediante la modulación de la vía del EGFR y de la ruta no canónica de NF-κB. En resumen, estos datos sugieren que la vía de señalización de EGFR podría ser considerada como una nueva diana terapéutica en el daño renal debido a su participación recurrente en el proceso inflamatorio y fibrótico en respuesta a diferentes estímulos en el riñón. El uso de bloqueantes de esta vía de señalización podría tener un alto valor terapéutico para el tratamiento de pacientes con ECR.

SUMMARY

Chronic kidney diseases (CKD) are characterized by persistent inflammation and progressive fibrosis leading to end stage renal disease. Several studies have demonstrated that CCN2, PTHrP and TWEAK, are key factors involved in the progression of CKD. EGFR is a membrane tyrosine kinase receptor expressed in the kidney. Previous data have described the role of EGFR in experimental renal fibrosis, but information about its potential role in the inflammatory process is scarce. The main aim of this thesis was to investigate the contribution of the EGFR pathway in experimental kidney damage, evaluating its role on inflammation and fibrosis, and the intracellular mechanisms involved in EGFR signalling. An additional goal was to increase the actual knowledge of renal pathology and improve the current therapeutic strategies. EGFR activation may occur by direct binding or by "transactivation", the latter process mediated by MMPs/ADAMs. The results of this study have shown that CCN2 is a new EGFR ligand that directly binds to this receptor and activates its signalling pathway, by an MMPs/ADAMs independent process. Furthermore, EGFR can be transactivated in the kidney by several factors involved in renal damage such as PTHrP and TWEAK, as described here for the first time. PTHrP, by its binding to the G coupled receptor PTH1R, can transactivate EGFR by 2 different mechanisms; PKC/MMPs activation or Src activation. TWEAK, through interaction with its receptor Fn14, activates ADAM17 that release HB-EGF and TGF-α ligands, leading to EGFR transactivation. Previous experimental studies suggest that EGFR pathway blockade could be an antifibrotic target for renal disease. *In vitro* studies showed that direct activation of EGFR by CCN2, and EGFR transactivation by PTHrP, could regulate epithelial mesenchymal transition, a process that contributes to renal fibrogenesis. *In vivo* studies, including the experimental models of renal damage mediated caused by systemic administration of CCN2 (IV) or TWEAK in mice, have demonstrated that activation of the EGFR pathway is associated with an inflammatory process in the kidney, which it is prevented by EGFR pharmacological blockade. In particular, TWEAK-induced EGFR transactivation and renal inflammation was blocked by ADAM17/EGFR inhibition, suggesting that the blockade of this axis could be a good therapeutic option for renal inflammatory diseases. Chronic renal disease is characterized by vitamin D deficiency. Several experimental data have shown that vitamin D receptor agonists (VDRAs) exert beneficial antinflammatory effects in CKD. In this thesis we have described that treatment with the VDRA Paricalcitol inhibited TWEAK-mediated EGFR transactivation and subsequent activation of intracellular mechanisms, including overexpression of proinflammatory factors and the infiltration of inflammatory cells into the kidney. The NF-κB plays a key role in the regulation of the inflammatory response. TWEAK is one of the few cytokines that activates both the canonical and non-canonical NF-κB pathway. Blockade of TWEAK-induced EGFR transactivation by different approaches, including Paricalcitol, inhibited the non canonical, but not the canonical, NF-κB pathway. These results described a novel antinflammatory mechanism of VDRAs that includes the modulation of the EGFR pathway and the inhibition of the noncanonical NF-κB2 pathway. In summary, all these data show that EGFR signalling pathway activation is involved in the modulation of inflammatory and fibrotic process in the kidney in response to different stimuli, and suggest that the blockade of EGFR signalling pathway could be considered as a new therapeutic target in renal diseases. Blockers of this signalling pathway could have a relevant therapeutic value for the treatment of CKD patients.

ÍNDICE

ABREVIATURAS

En algunos casos se ha conservado la correspondiente abreviatura en inglés debido a su frecuente utilización en el lenguaje científico.

α-SMA	α-actina de músculo liso
aa	aminoacidos
ADAMs	*Disintegrin and metalloprotease*
AGES	Productos finales de glicosilacion avanzada
Ang II	Angiotensina II
AP1	Proteína activadora 1
APOE	Apolipoproteína E
ARA II	Antagonistas de los receptores de angiotensina tipo I
BAF	*Barrier-to-autointegration factor*
Bcl-2	*B-cell lymphoma 2*
BMP	Proteínas morfogenéticas de hueso
CCL19	*Chemokine (C-C motif) ligand 19*
CCL21	*Chemokine (C-C motif) ligand 21*
CCN2	Factor de crecimiento del tejido conectivo
CXCL	*Chemokine (C-X-C motif) ligand*
CYR61	Proteína rica en cisteínas 61
DBP	Proteína de unión a vitamina D
DHR	Dominio de homología Rel
DNA	Ácido dexosiribonucleico
EGF	Factor de crecimiento epidérmico
EGFR	Receptor del factor de crecimiento epidérmico
ERC	Enfermedad renal crónica
ERK	Quinasa de regulación de señales intracelulares
ET-1	Endotelina-1
FGF	Factor de crecimiento de fibroblastos
Fn14	Factor de crecimiento de fibroblastos inducible-14
FSP-1	Proteina especifica de fibroblastos
GM-CSF	Factor estimulante de colonias de granulocitos y monocitos
GPCRs	Receptores acoplados a proteínas G
HB-EGF	Factor de crecimiento epidérmico de unión a Heparina
I-CAM-1	Molécula de adhesión intercelular-1
iECA	Inhibidores de la enzima convertidora de angiotensina
IFN-γ	Interferón- γ
IGF	Factor de crecimiento de tipo insulina
IL-10	Interleuquina-10
IL-1ra	Agonista del Receptor de interleuquina-1
IL-1β	Interleuquina-1β
IL-4	Interleuquina-6
IL-6	Interleuquina-6
IL-8	Interleuquina-8
ILK	Quinasa de union a integrinas
IP-10/CXCL10	*C-X-C motif chemokine 10*
JNK	Quinasa c-Jun N-terminal
LPA	Acido lisofosfatídico
LPR	Proteína de baja densidad
LPS	Lipopolisacárido bacteriano
MAPK	Proteínas quinasas activadas por mitógeno
MCP-1/CCL-2	Proteína quimioatrayente de monocitos-1
MEC	Matriz extracelular
MMPs	Metaloproteinasas
NEMO	*Modulador esencial deNF-κB*
NF-κB	Factor nuclear-κB
NIK	*Quinasa inductora de NF-kappaB*
PPARs	*Peroxisome proliferator-activated receptors*
NO	Oxido nitrico
NOV	Proteína sobreexpresada en nefroblastoma

OPN	Osteopontina
PAI-1	Inhibidor del activador del plasminógeno-1
PDGF	Factor de crecimiento derivado de plaquetas
PDGs	Productos de degradacion de la glucosa
PGE2	Protaglandina E2
PI3K	*PhosphatidylInositol 3-Kinase*
PKC	Proteína quinasa C
PLC	Fosfolipasa C
PTH	Hormona paratiroidea
PTH1R	Receptor de la hormaona paratiroidea
PTHrP	Proteina relacionada con la Paratohormona
RANTES/CCL-5	*Regulated on Activation, Normal T Expressed and Secreted*
ROS	Especies reactivas de oxigeno
RTKs	Receptores con actividad tirosina quinasa
RUNX2	*Runt-related transcription factor 2*
RXR	Receptor de acido retinoico
SiRNA	*Small interfering RNA*
SMAD	*Mothers against decapentaplegic homolog*
SPAK	*Stress-activated protein kinase*
SPARC	*Secreted protein acidic and rich in cysteine*
SRAA	Sistema Renina Angiotensina Aldosterona
sTNFr	Receptor soluble delFactor de necrosis tumoral
TEM	Transicion epitelio mesenquimal
TGF-α	Factor de crecimiento transformante- α
TGF-β	Factor de crecimiento transformante-β
TIMP-1	Inhibidor de metaloproteinasas de tejido
TME	Transicion mesenquima epitelio
TNFRSF	Superfamilia de receptores del factor de necrosis tumoral
TNF-α	Factor de necrosis tumoral-α
TPA	*Tetradecanoyl-phorbol-13-acetato*
TRAFs	Factores asociados a TNF
TrkA	Receptor del factor de crecimiento nervioso A
TWEAK	Citoquina inductora de apoptosis semejante a TNF
UUO	Obstrucción unilateral ureteral
V-CAM-1	*Vascular cell adhesion molecule-1*
VDR	Receptor de la vitamina D
VDRA	Agonista de los receptores de la vitamina D
VDRE	Elementos de respuesta a VDR
VEGF	Factor de crecimiento endotelial vascular
WISP	Proteínas secretadas inducidas por Wnt
ZO-1	*Tight junction protein-1*
-/-	*Knockout*

I. INTRODUCCÍON

1. SITUACIÓN ACTUAL DE LA ENFERMEDAD RENAL

La enfermedad renal crónica (ERC) es una patología progresiva que aparece comúnmente en la práctica clínica. El 15% de la población adulta mundial está afectada por ERC y su diagnóstico clínico depende del aumento de ciertos biomarcadores detectables en el momento en que la función renal disminuye, como son los niveles de creatinina en suero y la tasa de filtración glomerular. La ERC sigue siendo un problema significativo para los pacientes, sus familias y para la sociedad. En 2013, el informe Mundial acerca de la incidencia de Morbilidad, identificó la ERC como la principal causa de muerte no transmisible, cuyo índice de prevalencia ha aumentado más sobre la población general entre 1990 y 2013 (GBD 2013).

Estudios clínicos recientes han descrito que la ERC es un factor de riesgo independiente de la enfermedad cardiovascular crónica (Go et al., 2004, Ninomiya et al., 2005). Además, pacientes con ERC presentan un alto riesgo, entre 10 y 100 veces superior al de una persona sana, de desarrollar hipertensión y otras enfermedades cardiovasculares, como diabetes e hiperlipidemias, todas ellas asociadas a una elevada morbilidad y mortalidad (Wanner et al., 2005). El sistema Renina Angiotensina Aldosterona (SRAA) juega un papel fundamental en muchos de los cambios patofisiológicos que desencadenan la progresión de la enfermedad renal. El uso de agentes bloqueantes del SRAA son la primera opción en clínica. Estudios en pacientes con distintas nefropatías, incluidas la nefropatía diabética o la nefropatía hipertensiva, han observado efectos renoprotectores y beneficiosos derivados del uso de inhibidores de la enzima convertidora de angiotensina (iECA) o antagonistas de los receptores de angiotensina II (ARA II) (Brenner et al., 2001, Agodoa et al., 1997).

La ERC se caracteriza por una destrucción progresiva del parénquima renal y una pérdida de la funcionalidad de la nefrona que desemboca en un fallo renal terminal (Viau et al., 2010). Esta disfunción de la nefrona, desencadena procesos celulares y moleculares que pretenden promover el crecimiento compensatorio de la nefrona (Hostetter, 1995). En ciertos casos este proceso compensatorio llega a ser patológico, produciendo el desarrollo de lesiones renales y desembocando en enfermedad renal terminal (Terzi et al. ,1995, Kliem et al.,1996, Pillebout et al., 2003).

Actualmente uno de los mayores problemas en clínica, es el aumento de pacientes que progresan hasta insuficiencia renal terminal (Alcázar et al., 2008). La enfermedad renal terminal requiere en el 100% de los casos, una terapia renal de reemplazo como puede ser la diálisis (hemodiálisis/diálisis peritoneal) o el transplante renal. El 50% de los pacientes que se someten a diálisis no soportan mas de tres años con este tipo de terapias sustitutivas, de ahí la importancia del desarrollo de nuevas estrategias que sean capaces de restaurar la función del riñón o en su defecto, prevenir la progresión de la enfermedad renal, hecho que ha visto incrementado su incidencia sobre la población en las últimas tres décadas, haciendo que los pacientes puedan llegar a perder el 70% de su vida útil (Takase et al., 2014). Al comienzo del año 2000, la nefrología se encontraba entre las especialidades médicas con menor número de estudios clínicos llevados a cabo. Destacan las numerosas barreras que impiden el correcto diseño de fármacos para combatir el daño renal y que posteriormente puedan extrapolarse con éxito a la clínica. En este campo existe una gran desconexión entre la investigación básica en patología renal y la experimentación animal, ya que hay poca similitud entre los modelos animales de daño renal y los eventos patofisiológicos relevantes en la enfermedad renal humana (Sanz et al., 2013). Todo esto hace necesario promover la investigación básica en este área biomédica.

2. MECANISMOS CELULARES IMPLICADOS EN LA ENFERMEDAD RENAL

2.1. LA RESPUESTA INFLAMATORIA

La inflamación es un proceso tisular que conlleva eventos celulares y moleculares, cuya finalidad reside en la protección del tejido frente a agresiones de tipo fisicoquímico y biológico. La respuesta inflamatoria comienza con una serie de cambios vasculares como son la modificación del flujo y calibre vascular así como el incremento de la permeabilidad del vaso, seguidos de la extravasación de células inflamatorias provenientes de la circulación sanguínea al tejido dañado. Este último proceso está regulado por múltiples mediadores proinflamatorios (selectinas, moléculas de adhesión, integrinas y quimioquinas) que controlan los fenómenos implicados en la interacción de la célula inflamatoria con el endotelio vascular y su posterior integración y desplazamiento a la zona dañada del tejido (Springer 1995, Ebnet y Kaldjian 1996). En los focos de lesión tisular, las células residentes generan a su vez una fuerte producción local de quimioquinas que se difunden desde el epicentro inflamatorio al tejido circundante y fomentan el reclutamiento de neutrófilos, macrófagos, linfocitos T y B y mastocitos. Todas estas células infiltrantes presentes en el tejido dañado, secretan más factores de crecimiento y mediadores que amplifican la respuesta inflamatoria (Lee y Kalluri 2010, Ferenbach et al., 2007; Blank et al., 2007).

2.2. EL PROCESO INFLAMATORIO RENAL

La inflamación renal es un proceso complejo que refleja la respuesta local o sistémica frente a diferentes factores de estrés externos (uremia, estrés oxidativo, exposición a endotoxinas) y cuya finalidad es la reparación tisular. Esta respuesta se caracteriza por la infiltración glomerular e intersticial de células del sistema inmune con el objetivo de recuperar la funcionalidad del tejido, pero una persistencia de este proceso inflamatorio en el tiempo puede fomentar y contribuir al daño renal (Ucero et al., 2010). El tubulointersticio juega un papel clave en la progresión de la enfermedad renal ya que son todos estos factores externos los que activan las células tubulares causando cambios patológicos en el parénquima renal (Hodgkins y Schnaper, 2012).

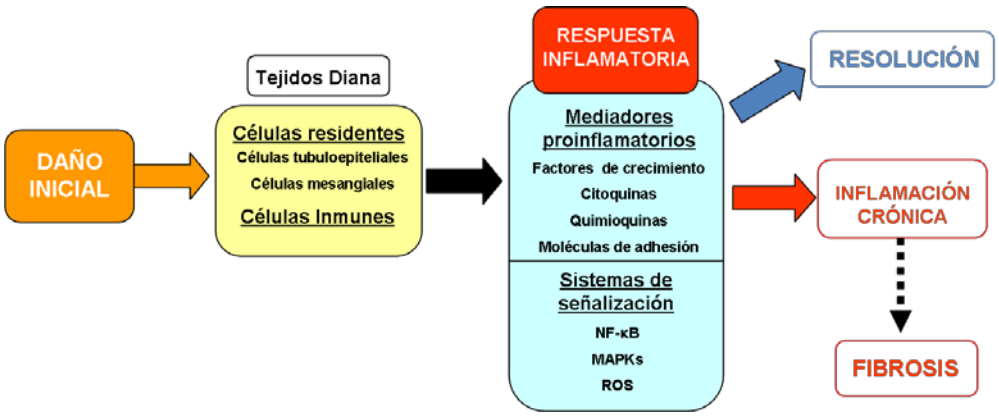

Figura 1: Mecanismos celulares y moleculares que se producen en respuesta a un daño renal.

Este proceso inflamatorio renal se caracteriza por la producción por parte de las células residentes, de diferentes mediadores inflamatorios como citoquinas proinflamatorias (IL-6, IL-1β, TNF-α) y antinflamatorias (IL-4, IL-10, sTNFr y IL-1ra), quimioquinas (MCP-1, RANTES, OPN), factores de crecimiento (CCN-2, TGF-β, EGF, PDGF, PTHrP), agentes vasodilatadores (VEGF, NO, protaglandinas) y vasoconstrictores (Ang II, ET-1) y especies reactivas de oxígeno (ROS). Todos estos factores se suman a la activación del sistema del complemento y a la expresión de moléculas de adhesión (I-CAM) y selectinas (V-CAM) (Jacobs et al., 2004, Schindler 2004), induciendo el reclutamiento de leucocitos al foco del daño incrementado así la inflamación residente en el tejido. La activación de estas respuestas se produce a través de diferentes vías de señalización, entre las que destacan la vía de NF-κB y la ruta de las MAPKs (Mezzano et al., 2001, Sanz et al., 2010, Ruiz-Ortega et al., 2002, Wolf 2004, Chung y Lan 2011, Guijarro y Egido 2001) **(Figura 1)**. La concentración de estas moléculas en el medio favorece la infiltración de células inflamatorias (macrófagos, neutrófilos y linfocitos) y la diferenciación y proliferación de miofibroblastos, lo que contribuye a la cronificación del proceso inflamatorio y, en último término, a la fibrosis (Lee y Kalluri 2010, Tang et al., 2011).

2.2.1. *Vía de señalización del Factor Nuclear-κB (NF-κB)*

NF-κB es un factor de transcripción que regula genes relacionados con inflamación, inmunidad, apoptosis, proliferación celular y diferenciación (Sanz et al. 2010). Un amplio espectro de estímulos relevantes para el daño renal activan NF-κB, incluyendo citoquinas, factores de crecimiento, patógenos, mediadores inmunes, proteinuria, estrés metabólico (alta glucosa, PDGs), estrés genotóxico y estrés mecánico (Chen et al 2009, Guijarro y Egido 2001). En respuesta a estos estímulos, NF-κB puede inducir la transcripción de genes tanto de respuesta temprana, como algunos reguladores negativos de la vía (IκBα, IκBε, A20) y citoquinas inflamatorias (IL-6, IL-8, MCP-1 y IP-10), así como genes de respuesta más tardía, entre los que se encuentran receptores celulares, moléculas de adhesión y quimioquinas, como RANTES. NF-κB no sólo actúa como activador de la expresión génica, también puede actuar como represor de la misma, a través de mecanismos como la inactivación de diferentes complejos proteicos o la modificación post-traduccional de las proteínas que forman parte de sus propios complejos (Sanz et al., 2010b).

Se han identificado 5 miembros de la familia de proteínas Rel como RelA/p65, RelB, cRel/Rel, NF-κB1 (p50; generado a partir de p105) y NF-κB2 (p52; generado a partir de p100), que pueden establecer potencialmente 15 homo/heterodímeros (Wan y Leonardo 2009). Las proteínas de la familia de NF-κB están constituidas por un región de homología Rel localizada en el extremo N-terminal de la proteína, de aproximadamente 300 aminoácidos que posee dominios de unión al DNA, y es responsable de su dimerización, interacción con las IκBs y de su localización nuclear. Además, la región DHR contiene una señal de localización nuclear que facilita la translocación del NF-κB al núcleo. Los dímeros de NF-κB se unen al promotor de gran variedad de genes a través de sitios κB que poseen en su secuencia (Wan y Leonardo 2009).

El dímero RelA/p50 es el más abundante y mejor caracterizado de todos los miembros de la familia. Estos dímeros de NF-κB se mantienen inactivos en el citoplasma celular unidos a las proteínas IκB. Esta familia de proteínas IκB esta constituida por 8 miembros (IκBα, IκBβ, IκBγ, IκBδ, IκBε, Bcl-3, y los precursores p100 y p105) que están caracterizados por la presencia de 5 repeticiones de tipo anquirina que se unen al dominio de dimerización de NF-κB impidiendo su traslocación al núcleo (Karin y Neriah 2000; Senftleben et al. 2001).

Existen 2 vías diferentes de activación para NF-κB, la vía de activación clásica/canónica y la vía de activación alternativa/no canónica **(Figura 2)**. La activación de NF-κB a través de la vía canónica es una activación rápida en respuesta a un amplio rango de estímulos. Esto induce la activación del complejo IKK, constituido por dos subunidades catalíticas IKKα e IKKβ, y la subunidad regulatoria IKKγ/NEMO (Hayden y Ghosh 2004). Este complejo induce la fosforilación de IKKβ y la degradación por ubiquitinación de la subunidad inhibitoria IκB vía proteasoma 26S, lo cual permite la fosforilación de la subunidad RelA y la traslocación al núcleo del complejo RelA/p50, para unirse al DNA y activar la transcripción de diferentes genes diana.

La vía no canónica de activación de NF-κB en cambio se produce a un tiempo más tardío. Este proceso alternativo comienza con la activación de la quinasa NIK, el reclutamiento de IKKα para formar el complejo dimérico IKKα/IKKα que se fosforila y causa la degradación, a través del proteasoma, de la subunidad p100 perteneciente al complejo NF-κB2 formado por p100/p52 (Hayden y Ghosh 2004). Esta degradación permite la formación de los complejos RelB/p52 que se liberan y migran al núcleo para unirse al DNA. Sin embargo, p100 también se une e inhibe RelA, c-Rel, y p50, subunidades que cuando son procesadas generan los complejos p52/RelA, p52/c-Rel, o p52/p50 (Sanz et al., 2010). La activación de esta vía permite la transcripción de un grupo de genes distintos a los regulados a través de la vía clásica, entre los que se encuentran quimioquinas como CCL-21, CCL-19, CXCL13; CXCL12 y BAF (Dejardin et al. 2002) **(Figura 2)**. Sin embargo, la regulación de las dianas de la vía no canónica en células renales no ha sido muy estudiada hasta ahora.

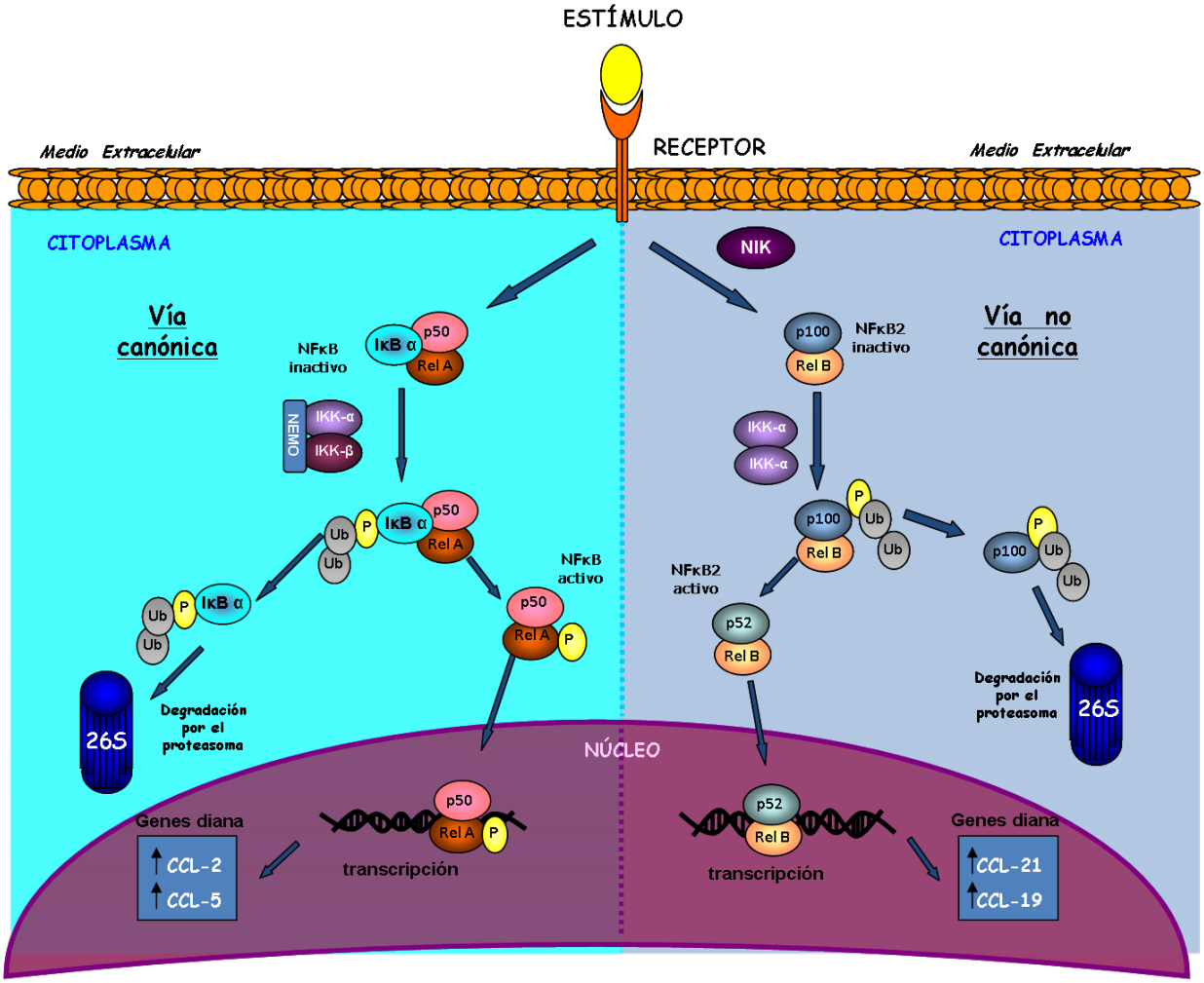

Figura 2: Vías de señalización del factor de transcripción NF-κB: Activación canónica y no canónica.

NF-κB es un factor de transcripción que está implicado en la patogénesis de multitud de enfermedades inflamatorias como la artritis reumatoide (Gregersen et al., 2009), enfermedad de Bowel (Hollenbach et al., 2004), ateroesclerosis (Sun et al., 2013), y cáncer (Karin y Greten, 2005). También se ha descrito su participación en la nefropatía diabética humana y en la enfermedad glomerular, existiendo evidencias histológicas de su activación (Mezzano et al., 2004, Sanz et al., 2010). En la enfermedad renal experimental, la vía de NF-κB se encuentra activa en procesos tales como isquemia reperfusión, hipertensión, obstrucción, daño renal agudo séptico/tóxico y patologías de base inmune (Morin et al., 2014, Guijarro y Egido 2001, Sanz et al., 2010b, Sanz et al., 2008, Ruiz-Ortega et al., 1998, Ruiz-Ortega et al., 2001, Lopez-Franco et al., 2002). Estudios experimentales muestran que el bloqueo directo de la vía canónica de NF-κB, mediante diferentes estrategias como la sobreexpresión del IκB, el uso de péptidos bloqueantes de la traslocación nuclear del Rel A (SN50) e inhibidores farmacológicos (Parthenolide), o el bloqueo indirecto mediante diversos compuestos, entre los que destacan los iECAs, ARA II, glucocorticoides y antioxidantes, previenen el daño renal (Guijarro y Egido 2001, Barnes y Karin 1997, Gruden et al., 2005,Esteban et al., 2004, Tomita et al., 2000)

Actualmente existen pocos estudios experimentales acerca de la vía no canónica de NF-κB. La fosforilación de la quinasa NIK, componente desencadenante de la vía, se encuentra aumentada durante procesos de isquemia-reperfusión, pero su comportamiento en otros procesos asociados al daño renal no ha sido analizado hasta ahora (Loverre et al., 2004). Las toxinas uremicas p-cresil-sulfato e indoxil-sulfato inducen la expresión de NF-κB2 en un 50-80% en células tubuloepiteliales proximales en cultivo (Sun et al., 2013). En modelos de nefropatía diabética, proteínas como NIK y RelB están aumentadas (Starkey et al., 2006), y en modelos de daño renal agudo por toxicidad, se ha observado un aumento de la expresión proteica en el riñón de RelB y p52, además de un incremento en la expresión génica de sus genes diana (Sanz et al., 2010).

2.2.2. *Vía de señalización de las quinasas activadas por mitógeno (MAPKs): Quinasa ERK*

Las quinasas activadas por mitógeno (MAPKs) son un grupo de serina/treonina-quinasas que están implicadas en la dirección y transmisión de señales celulares activadas por estímulos extracelulares, que incluyen hormonas, mitógenos, factores tróficos y de crecimiento, citoquinas inflamatorias y diversas formas de estrés celular (osmótico, redox, radiación, etc.). Además participan en el control de la expresión génica por receptores de membrana, regulando procesos tan importantes como proliferación, diferenciación y transformación celular, desarrollo, apoptosis e inflamación crónica (Lei et al., 2014).

Estas quinasas están organizadas en módulos constituidos por una proteína G y tres proteínas quinasas que actúan en cascada y se fosforilan en residuos tirosina o treonina (MAPK, una MAPK activadora (MAPKK) y una MAPKK activadora (MAPKKK)) que a su vez son reguladas por proteínas G o por fosforilación por MAPKKK quinasas (MAPKKKK) (Bagley et al., 2010; Cargnello y Roux 2011). Existen diferentes subgrupos de MAPKs entre los que se encuentran el grupo ERK (ERK1-5), c-Jun (JNK/SAPK) y p38 MAPK (p38α/p38β/p38γ/p38δ). Entre todas estos grupos el mejor caracterizado es el de las ERK. Las ERK1/2 son quinasas fosforiladas por MEK1/2 y éstas por MAPKKK de la familia Raf entre otras, las cuales son activadas por la proteína G monomérica Ras, en respuesta a hormonas y factores de crecimiento. La activación de las ERKs estimula la puesta en marcha del ciclo celular regulando

proliferación e hipertrofia celular, y participando en procesos fibróticos e inflamatorios (Bagley et al., 2010). Las ERK3/4 son consideradas atípicas ya que su activación carece de residuos tirosina y presentan motivos Ser-Glu-Gly. En concreto ERK3 regula la proliferación celular, progresión y diferenciación del ciclo celular. La función de ERK4 no se conoce hasta ahora (Cargnello y Roux 2011). En cambio ERK5, también denominado BMK, se activa por varios estímulos como estrés oxidativo y factores de crecimiento, siendo esencial en desarrollo embrionario temprano, desarrollo vascular normal y supervivencia celular.

3. PROCESO FIBRÓTICO

La fibrosis es un proceso reparador que se activa en respuesta a un daño con el fin de preservar la arquitectura del tejido y su integridad funcional. Sin embargo, la presencia de estímulos dañinos de forma crónica puede causar la desregulación del proceso normal de reparación y desembocar en una acumulación de matriz extracelular (MEC), principalmente de los componentes fibronectina y colágeno I, III y IV, lo que conduce a la fibrosis (Shi-Wen et al., 2008). Este proceso fibrótico conlleva diferentes eventos, comenzando por una infiltración inflamatoria intersticial y activación de los fibroblastos y las células mesangiales (Lee y Kalluri 2010). En condiciones patológicas, tanto las células túbuloepiteliales como las células endoteliales, pueden sufrir transición epitelio/endotelio-mesénquimal (TEM), convirtiéndose en miofibroblastos productores de MEC y contribuyendo a la fibrosis renal y a la progresión de la enfermedad (Roberts et al., 2006). En este proceso participan diversos factores, entre los que destacan TGF-β, CCN-2, FGF, IL-1β, AGES y Ang II (Utsugi et al., 2003;Carvajal et al., 2008, Burn et al., 2006 , Liu et al., 2008 , Shi-Wen et al., 2008, Zhang et al., 2005).

3.1. TRANSICIÓN EPITELIO MESENQUIMAL (TEM)

Las células epiteliales son células polarizadas cuya superficie basal se asienta en una membrana basal y su parte apical está dirigida a la luz de una cavidad. Lateralmente, las células interactúan con las células adyacentes a través de uniones adherentes, desmosomas y uniones estrechas, formando un pavimento de células en la membrana basal (Fragiadaki y Mason 2011).

Durante el proceso de embriogénesis, las células epiteliales se transforman en células mesenquimales dando lugar a un proceso denominado transición epitelio-mesénquimal tipo 1 (TEM) (Kalluri y Weinberg 2009). Durante el desarrollo embrionario las células mesenquimales son capaces de migrar a través de la MEC y posteriormente, diferenciarse hacia los diferentes linajes celulares mesenquimales, incluyendo los fibroblastos. El proceso inverso, la transición-mesénquima epitelio (TME), también se produce durante el desarrollo embrionario y se requieren varias rondas de TEM/TME para generar un embrión con tejidos y células especializadas (Thiery et al., 2009).

Diversos estudios han propuesto que la TEM también ocurre durante la reparación de los tejidos y la fibrosis (TEM Tipo 2) y durante la progresión y metástasis de procesos cancerosos (TEM Tipo 3) (Kalluri y Weinberg 2009). Los tipos 2 y 3 comparten muchas características comunes con el Tipo 1, por ejemplo, la pérdida de las uniones intercelulares entre las células epiteliales, la separación de la membrana basal, la pérdida de marcadores de fenotipo epitelial y la adquisición de marcadores mesenquimales, además de la migración celular. Sin embargo, también presentan algunas características distintas. La TEM Tipo 1 se produce en etapas específicas durante la

embriogénesis, en cambio la TEM tipo 2 y 3 tienen lugar durante periodos prolongados, y conducen a la fibrosis o metástasis (Kalluri y Weinberg 2009).

Se han descrito distintas teorías acerca del origen de esas células mesenquimales, incidiendo en la posibilidad de que los miofibroblastos puedan derivar de diferentes tipos celulares: 1) de fibroblastos intersticiales renales cuya función fisiológica es mantener la estructura del riñón regulando la homeostasis de la MEC (Qi et al., 2006), 2) de fibroblastos perivasculares y pericitos (Lin et al., 2008); 3) de la diferenciación de células madre derivadas de células de la médula ósea (Broekema et al., 2007); 4) y de la transición de células endoteliales o células epiteliales tubulares a células mesenquimales (TEM) (Zeisberg et al., 2008, Strutz et al., 1995, Iwano et al., 2002). Diversos grupos sugieren que el proceso de TEM en el riñón es un mecanismo que contribuye a la fibrosis renal (Iwano et al., 2002, Zeisberg et al., 2008).

Los túbulos renales están compuestos por células epiteliales que se asientan en una membrana basal tubular, rodeada por una pequeña cantidad de tejido intersticial. La liberación prolongada de factores estimulantes para las células residentes, incluidos; factores de crecimiento como TGF-β1 (Inazaki et al., 2004, Wang et al., 2010) y CCN2 (Yokoi et al., 2004), citoquinas como IL-1β (Vesey et al., 2002), oncostatina M (Nightingale et al., 2004), Ang II (Ishidoya et al., 1995), proteasas (Cheng et al., 2006; Zhang et al., 2007), PAI-1 (Matsuo et al., 2005) y AGES (Burns et al., 2006), además de la infiltración de células inflamatorias locales, conduce a estas células a experimentar una serie de cambios caracterizados por la pérdida de marcadores de fenotipo epitelial (E-cadherina, ZO-1, citoqueratina o la traslocación nuclear de β-catenina) y la adquisición de marcadores mesenquimales (FSP-1, α-SMA, Vimentina, ILK o la traslocación al núcleo de Snail). Este cambio induce a su vez la pérdida de adhesión epitelial, reorganización de citoesqueleto, producción excesiva de MEC en el intersticio (principalmente colágenos y fibronectina), interrupción de la membrana basal tubular y, finalmente migración e invasión del intersticio (Liu 2004) **(Figura 3).**

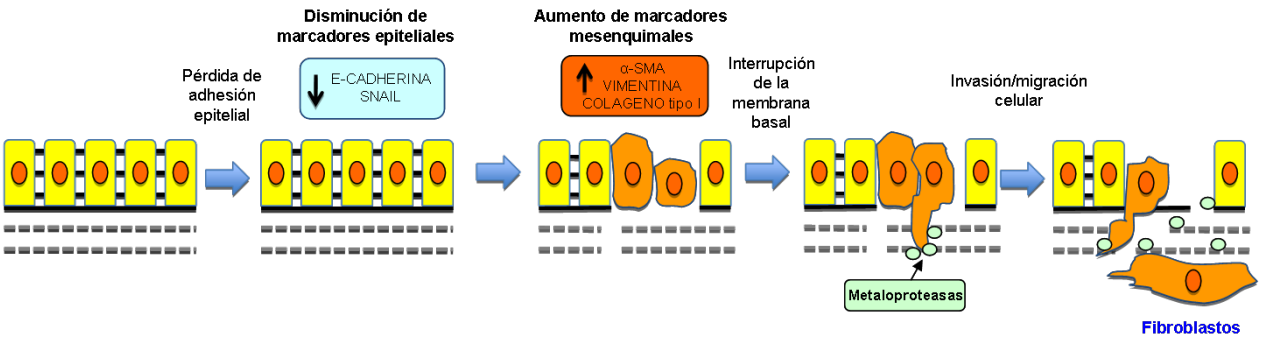

Figura 3: Etapas de la Transición Epitelio-Mesenquimal.

4. RECEPTORES DE MEMBRANA

La unión de neurotransmisores, hormonas o factores de crecimiento (ligandos) a sus receptores de membrana, produce cambios bioquímicos en el interior de la célula, que conducen a una respuesta específica para el estímulo inicial. Existen diferentes grupos de receptores de membrana, definidos por sus mecanismos de transducción de señales, como son los receptores ionotrópicos, los receptores acoplados a proteínas G (GPCRs) y los receptores con actividad tirosina quinasa (RTKs).

El proceso de activación de estos receptores puede producirse de dos maneras: 1) mediante activación directa ligando-receptor en la que no participan ningún otro tipo de proteína, o 2) activación indirecta. Este último proceso puede desencadenarse por estímulos fisiológicos y no fisiológicos.

Entre los estímulos no fisiológicos destacan la hiperosmolaridad, el estrés oxidativo, el estrés mecánico, la luz ultravioleta y la radiación γ, que activan este proceso por inactivación de determinadas fosfatasas que antagonizan la actividad quinasa intrínseca del receptor, permitiendo la autofosforilación del mismo (Fisher et al., 2003). La activación por estímulos fisiológicos, como quimioquinas, moléculas de adhesión y factores de crecimiento, que se produce por interacción previa con sus receptores específicos (GPCRs o no) es un proceso denominado "transactivación" que más adelante será tratado en profundidad.

4.1. RECEPTORES ACOPLADOS A PROTEÍNAS G (GPCRs)

Los GPCRs son proteínas que conforman la familia más grande (~1000 diferentes miembros) de receptores de membrana involucrados en la transmisión de señales. Estos receptores están constituidos por siete dominios transmembrana, tres asas intracelulares y tres extracelulares, además presentan regiones amino terminales (7-595aa), localizadas en la porción extracelular del receptor, y regiones carboxilo terminales (12-359aa), localizadas en la porción intracelular, que permiten regular la funcionalidad del receptor, mediante glicosilación y fosforilación respectivamente (Lefkowitz 1998). Por otra parte, los GPCRs se encuentran implicados en la activación de un gran número de cascadas de señalización, que en última instancia modifican la función de canales iónicos, receptores y enzimas, así como la transcripción génica.

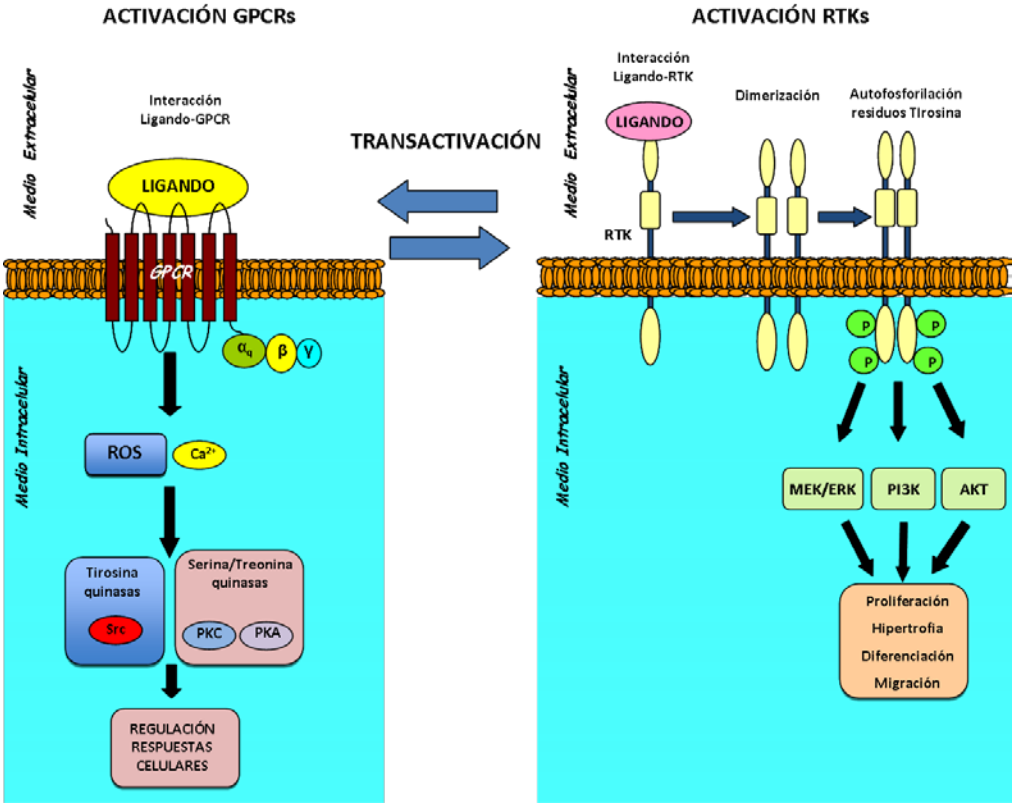

Figura 4: Sistemas de activación de receptores de membrana: Receptores acoplados a proteínas G (GPCRs) *vs* receptores tirosina quinasa (RTKs).

La señalización mediada por GPCRs se inicia al formarse el complejo ligando-receptor-proteína G, conocido también como "complejo ternario" en el que el receptor existe en dos estados distintos; uno inactivo y otro activo (este último unido a proteínas G). Una vez adquirido su estado activo, el receptor puede interaccionar con el ligando, sufriendo cambios conformacionales que dan lugar a la activación de cascadas de señalización capaces de modular la funcionalidad celular (Sánchez-Lemus y Arias-Montaño 2004). Se ha descrito que la activación de GPCRs desemboca en la activación de RTKs. Este proceso ha sido denominado "transactivación", y depende de señales intracelulares, en las que participan tanto las proteínas G, proteínas quinasas, como la PKC y tirosinas quinasas, diferentes de los propios RTKs (Van Corven et al.,1993 ,Koch et al., 1994). **(Figura 4)**

4.2. RECEPTORES TIROSINA QUINASA (RTKs)

Los RTKs son proteínas de membrana con un dominio transmembrana y dominios catalíticos con actividad tirosina quinasa. La porción amino terminal de estas proteínas está localizada en la región extracelular y presenta dominios ricos en cisteínas que participan de manera crucial en la formación de dímeros (Hurwitz et al., 1991), además de sitios de glicosilación que pueden regular la unión del ligando al receptor. El extremo carboxilo terminal se localiza en la región intracelular y presenta un dominio con actividad tirosina quinasa que contiene residuos tirosina que pueden ser fosforilados. La señalización mediada por RTKs se inicia al producirse la unión del ligando al receptor, que produce cambios conformacionales en el receptor fomentando su dimerización y autofosforilación en sus residuos tirosina. Estos residuos sirven como sitios de activación para proteínas con dominios SH2 o como sitios de unión para proteínas adaptadoras que son necesarias para continuar la señalización intracelular (Alberts et al., 1994) **(Figura 4).** Los RTKs son activados de manera primaria por factores de crecimiento como EGF, VEGF, PDGF e IGF (Alberts et al., 1994), y su estimulación inicia cascadas de señalización que regulan eventos esenciales para la proliferación, migración, crecimiento y diferenciación celular, en las que participa de manera relevante la cascada de señalización MAPKs (Sanchez Lemus y Arias-Montaño 2004).

4.3. RECEPTOR DEL FACTOR DE CRECIMIENTO EPIDÉRMICO (EGFR)

El receptor del factor de crecimiento del tejido epidérmico (EGFR; HER1; ErbB1) es una glicoproteína transmembrana de 1186 aa (180 KDa) que pertenece a la familia de receptores tirosina quinasa ErbB, de la que forman parte otros miembros, como HER2/neu (ErbB2), HER3 (ErbB3), y HER4 (ErbB4). El EGFR está constituido por un dominio extracelular con regiones ricas en cisteínas (responsables de la unión al ligando), un dominio transmembrana, y un dominio intracelular con regiones tirosina quinasa (dominio de activación) (Holbro y Hynes 2004). Este receptor puede activarse de forma directa o mediante transactivación.

El primer paso para la activación directa del EGFR comienza con la unión del ligando al receptor. Hasta el momento se conocen 11 ligandos que interaccionan con esta familia de receptores, entre los que se encuentran; EGF (ligando canónico de la vía), TGF-α, HB-EGF, amfiregulina, betacelulina, epiregulina, epigen y cripto (Marquart et al., 1984, Shoyab et al., 1989; Higashiyama et al., 1991, Shing et al., 1993, Toyoda et al., 1995). La afinidad del EGFR por un ligando u otro en su proceso de activación depende del tejido en el que se encuentre, y según el ligando con el que interaccione, puede generar distintas respuestas celulares. En general, los ligandos del EGFR se localizan como

precursores transmembrana inactivos, que para poder unirse a su receptor necesitan ser sometidos a un procesamiento proteolítico y liberarse como ligandos solubles al medio extracelular. Este procesamiento proteolítico es llevado a cabo por metaloproteasas/disintegrinas de la familia de las ADAMs, claves en el proceso de transactivación, entre las que destacan ADAM9/10/12/15/17 y 19 (Melenhorst et al., 2008) **(Figura 5).**

Una vez unido el ligando al EGFR, el receptor sufre cambios conformacionales que inducen la formación de homo- o heterodímeros. Como consecuencia de esto, el dominio intracelular se activa fosforilándose en sus residuos tirosina y fomentando la autofosforilación de estos mismos residuos en su homólogo. Los residuos fosforilados sirven a su vez como sitio de unión para ciertas moléculas que presentan dominios de homología Src y cuya unión desemboca en distintas cascadas de señalización (Yu et al., 2002).

La activación indirecta del EGFR, también denominada transactivación, se desencadena por la unión de moléculas como Ang II, trombina, ET-1, TPA, LPA, TGF-β y TNF-α a sus receptores específicos (Hobbs et al., 2011, Ebi et al., 2010, Daub et al., 1997, Daub et al., 1996, Hackel et al 1999, Luttrell et al., 1999). Tras esta unión con sus receptores, se induce la liberación de segundos mensajeros, como el Ca^{2+} intracelular, ROS y determinadas proteínas quinasas como la PKC (Kodama et al., 2002, Soltoff 1998, Tsai et al., 1997), que inducen la activación de metaloproteasas/disintegrinas de la familia de las ADAMs. En algunos casos, se ha descrito también el papel de ciertas quinasas intracelulares que son capaces de activar independientemente de MMPs al EGFR, como es el caso de la quinasa Src (Bokemeyer et al., 2000, Shah et al., 2003, Luttrell et al., 1997, Zhuang S 2008) **(Figura 5).**

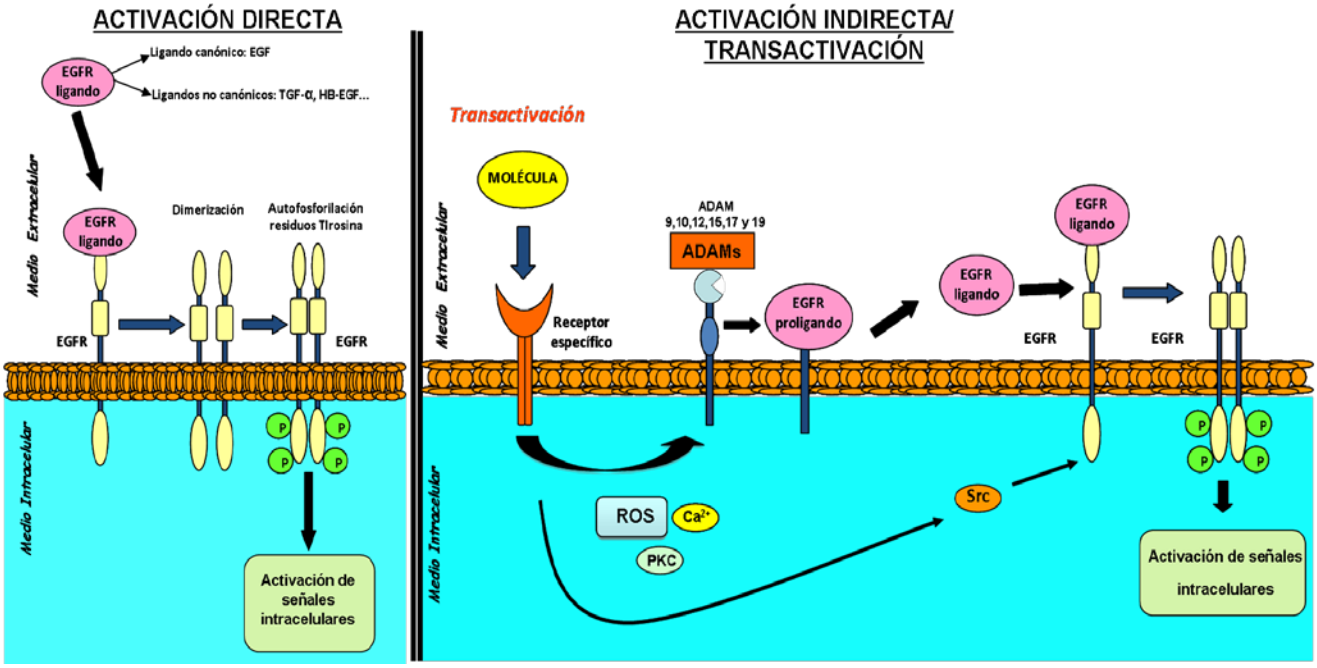

Figura 5: Tipos de activación del EGFR: Activación directa/ligando-receptor y activación indirecta/Transactivación.

4.4. ADAMs : PROTEÍNAS CLAVE EN LA TRANSACTIVACIÓN DEL EGFR

Las ADAMs son una familia de 23 glicoproteínas localizadas en la superficie celular, que contienen un dominio disintegrina y un dominio metaloproteasa, lo que hace que tengan características de moléculas de adhesión y de proteasas (Schlöndorffff y Blobel 1999). Las primeras ADAMs descritas estaban implicadas en funciones de

reproducción, fundamentalmente en la espermatogénesis y la unión del esperma al óvulo (ADAM1 y ADAM2). Recientemente, se ha descrito el papel de diferentes ADAMs como ADAM9/10/12/15/17 y 19 en la liberación y/o activación de proteínas de la superficie celular, entre las que se encuentran diversos ligandos del EGFR (Schlöndorff y Blobel 1999, Huovila et al., 2005). Dependiendo del tipo de tejido, diferentes ADAMs pueden estar implicadas en el proceso de liberación de los ligandos del EGFR. Así, en el riñón el proceso de transactivación del EGFR inducido por Ang II está mediado por ADAM17 (Lautrette et al., 2005), mientras que en el corazón se produce a través de ADAM12 (Asakura et al., 2002).

Estas glicoproteínas ADAMs se sintetizan en el aparato de Golgi y posteriormente, por acción de la proteasa furina, sufren un cambio conformacional que induce su activación. En su forma activa, son transportadas hasta la membrana plasmática donde ejercen su actividad de corte sobre los precursores inactivos de los ligandos del EGFR. Una vez estos precursores son procesados proteolíticamente, se liberan al medio extracelular y pueden interaccionar con el EGFR y activar esta ruta de señalización (Melenhorst et al., 2008). Dependiendo del tipo de ligando del EGFR que se libere y de la tirosina fosforilada en el EGFR, se activarán distintos mecanismos intracelulares entre los que destacan la activación de las MAPKs y de la PI3K. Estas vías de señalización traducen señales al núcleo, modulando la actividad de factores de transcripción como c-jun, c-fos, c-myc, y NF-κB y regulando la transcripción de genes y síntesis de determinadas proteínas (Holbro y Hynes 2004).

4.5. EGFR EN LA PATOLOGÍA RENAL

El EGFR es un receptor de membrana que está presente en el riñón, y cuya expresión se localiza en glomérulo, y túbulointersticio renal humano (Yoshioka et al., 1990), además se ha descrito su papel clave en la homeostasis electrolítica renal (Melenhorst et al., 2008). En distintos tipos de patologías renales, como glomerulonefritis, rechazo del transplante o poliquistosis renal entre otras; se ha observado un aumento de la expresión del EGFR (Sis et al., 2004, Nakopoulou et al., 1994, Takemura et al., 1999). El papel del EGFR en la patología renal es un poco contradictorio ya que se han descrito acciones tanto beneficiosas como deletéreas. Diferentes estudios experimentales han demostrado que el bloqueo del EGFR puede ser una importante herramienta en la enfermedad renal progresiva al presentar efectos beneficiosos sobre la fibrosis (Flamant et al., 2012). Algunos estudios han demostrado que los ligandos del EGFR, como EGF y HB-EGF pueden inducir regeneración renal, al ser capaces de aumentar la proliferación de células tubulares o inhibir su apoptosis, tanto en estudios *in vitro* como en la administración *in vivo* en fases tempranas en modelos experimentales de daño renal (Chevalier et al., 1999, Humes et al 1989, Singh et al., 2007, Zhuang et al., 2004, Wassef et al., 2004, Zhuang et al 2008, Flamant et al., 2012). Todo esto muestra la complejidad de la vía de señalización del EGFR y la importancia de profundizar en su estudio.

5. FACTORES CLAVES IMPLICADOS EN EL DAÑO RENAL

5.1 FAMILIA DE PROTEINAS CCN

La MEC es conocida como un regulador dinámico y multifuncional del comportamiento celular (Aszodi et al., 2006). Esta puede interaccionar y modular la viabilidad y actividad de factores de crecimiento, citoquinas, quimioquinas y enzimas extracelulares. Además, las proteínas de matriz pueden unirse directamente a receptores de

la superficie celular para desencadenar la activación de cascadas de señalización que regulan respuestas celulares. Dentro de este grupo de proteínas de matriz se encuentran proteínas matricelulares, entre las que destacan trombospondina, SPARC, hevina, osteopontina, tenascina C/X y la familia de proteínas CCN (Cheng y Lau 2009), cuya función primordial es modular respuestas celulares frente a factores medioambientales (Bornstein y Sage 2002).

Estudios recientes han demostrado la importancia de las proteínas CCN en la regulación del desarrollo embrionario, y en la etapa adulta en procesos como inflamación, reparación de daño, fibrosis y cáncer. Esta familia de proteínas CCN fueron identificadas como proteínas secretables, cuya síntesis está regulada por factores de crecimiento y oncogenes. Las primeras proteínas CCN identificadas fueron CYR61 (proteína rica en cisteína 61; CCN1) (O'Brien et al., 1990), CTGF (factor de crecimiento del tejido conectivo; CCN2) (Bradham et al., 1991), y NOV (proteína sobreexpresada en nefroblastoma; CCN3) (Joliot et al., 1992). CCN4 (WISP1), CCN5 (WISP2), y CCN6 (WISP3) fueron identificadas posteriormente como proteínas secretadas inducidas por Wnt (Pennica et al., 1998). Desde 2003, todas estas proteínas se denominan CCN1-6 independientemente de su nomenclatura de origen (Brigstock 2003).

Las proteínas CCN presentan una estructura modular con cierta homología con otras proteínas reguladoras. Están constituidas por un péptido secretor en el extremo N-terminal, seguido de 4 módulos funcionales (Bork 1993): 1) dominio de unión al factor de crecimiento similar a la insulina (IGF), con la secuencia de unión conservada Gly-Cys-Gly-Cys-Cys-X-XCys que se localiza dentro de la región amino-terminal de todas las proteínas de unión a IGF (Albiston y Herington 1990, Binkert et al., 1989, Brinkman et al., 1988); 2) dominio del factor Von Willebrand tipo C (Mancuso et al., 1989); 3) dominio trombospondina-1 (Holt et al., 1990); y 4) dominio C-terminal (Hoshijima et al., 2006). Las regiones que existen entre todos los módulos son susceptibles de ser separadas mediante proteólisis (Perbal y Takigama 2005), lo que puede dar lugar a moléculas individuales con actividad biológica (Brigstock 1999). La presencia de estas proteínas "truncadas" se ha asociado con etapas específicas del desarrollo y situaciones patológicas (Perbal 2001a). La existencia de estas secuencias proteolizables constituiría, por tanto, un tipo de regulación adicional de la actividad de estas proteínas (Perbal 2001b, Perbal 2004).

5.1.1. Factor de crecimiento del tejido conectivo (CCN2).

CCN2, también conocido como CTGF, es una proteína secretable, rica en cisteínas, con un peso molecular de 38 kDa, que fue identificada en el medio condicionado de células endoteliales de vena de cordón umbilical (Bradham et al., 1991). CCN2 es una proteína matricelular y modular **(Figura 6)**. Entre el módulo 2 y 3 existe una región bisagra que puede ser procesada por múltiples proteasas, incluidas las MMPs 1/2/3/7/9 y 13, generando dos porciones proteícas (una con el dominio N-terminal y otra con el dominio C-terminal), ambas con actividad biológica. También puede ser proteolizada por elastasa y plasmina, que pueden escindir los módulos individuales para producir cuatro fragmentos (Hashimoto et al., 2002). Además, se ha observado que *in vitro* MMP2 procesa CCN2 generando el fragmento C-terminal de 10-12 kDa (denominado en esta tesis CCN2(IV)). En fluidos biológicos y en el medio de células en cultivo, se ha descrito la presencia de CCN2 en sus diferentes formas: molécula completa CCN2, fragmento C-terminal (CCN2(IV)) y el fragmento N-terminal (Winter et al., 2008, Brigstock et al., 1997). Sin embargo, los efectos biológicos *in vivo* de CCN2 y de sus fragmentos no ha sido investigado en profundidad.

CCN2 es un gen de desarrollo que no se expresa en los tejidos adultos pero se induce en condiciones patológicas, incluyendo escleroderma, fibrosis pulmonar, fibrosis hepática (Perbal 2004, Leask y Abraham 2006;

Wang et al., 2001) y multitud de enfermedades renales, incluida la nefropatía diabética (Ito et al., 1998, Riser et al., 2000; Makino et al., 2003). Diversos estudios han evaluado en diferentes enfermedades renales crónicas los niveles de CCN2 en orina y/o suero, mediante técnicas de ELISA, usando anticuerpos que reconocen las diferentes partes de la molécula. Así, han observado cambios en los niveles de la molécula total de CCN2 (O'Seaghdha et al., 2011, Nguyen et al., 2006), de su fragmento N-terminal (Nguyen et al., 2006, Slagman et al., 2011), y del fragmento C-terminal (Riser et al., 2003, Tam et al., 2009, Bao et al., 2008). Según estos datos se ha postulado que CCN2 podría ser utilizado como un biomarcador de progresión de daño en la nefropatía diabética humana y en otras ERC (Phanish et al., 2010, O'Seaghdha et al., 2011; Nguyen et al., 2006, Slagman et al., 2011, Riser et al., 2003, Tam et al., 2009, Bao et al., 2008). Además, se ha sugerido que CCN2 también podría usarse también como biomarcador de la disfunción cardiaca en pacientes con fibrosis miocárdica y fallo cardiaco crónico (Koitabashi et al., 2008).

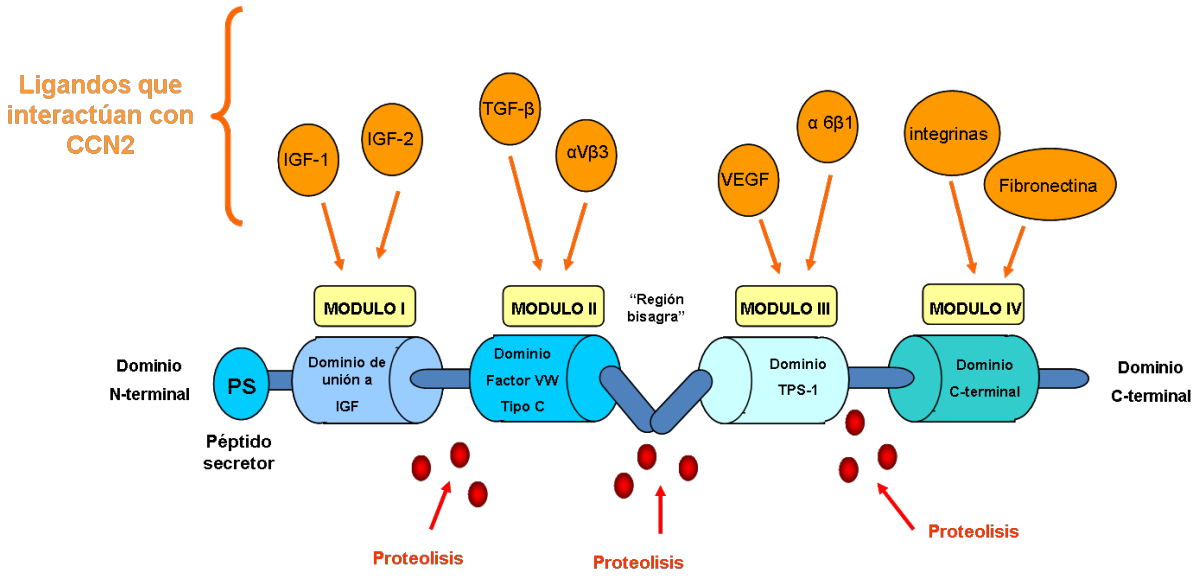

Figura 6: Estructura de CCN2. Módulos que lo componen y ligandos con los que interacciona con distinta afinidad.

CCN2 tiene un papel importante en múltiples procesos celulares como desarrollo, diferenciación, proliferación celular, remodelación de la MEC y angiogénesis (Phanish et al., 2010). Según el tipo celular, una gran variedad de factores y moléculas están implicadas en la inducción y regulación de la expresión de CCN2, incluidos agonistas de GPCRs, como Ang II, factores de crecimiento como TGF-β, BMP, VEGF, IGF, GM-CSF, IL-4, altas concentraciones de glucosa, AGEs, hipoxia, estrés mecánico y estrés oxidativo (Babic et al., 1999, Chen et al., 2003, Hoshijima et al., 2006, Liu et al., 2007, Murphy et al., 1999, Park et al., 2001, Rimon et al., 2008, Rishikof et al., 2002, Zhou et al., 2004, Leask y Abraham 2006). Clásicamente CCN2 ha sido descrito como un mediador profibrótico. El primer estudio llevado a cabo acerca de las propiedades profibróticas de CCN2 fue desarrollado por Gary Grotendorst en 1997 (Grotendorst 1997). En este estudio se demostró que CCN2 actúa como un mediador de las acciones profibróticas de TGF-β. Posteriormente, se ha descrito que sólo la administración conjunta de CCN2 y TGF -β, pero no de cada factor individualmente, induce fibrosis en la piel (Holmes et al., 2001). La sobreexpresión de CCN2 ha sido muy estudiada en distintas enfermedades fibróticas humanas, y se ha localizado temporalmente y espacialmente coincidente con las áreas fibróticas (Phanish et al., 2010, Leask et al., 2009). En células mesangiales y fibroblastos, CCN2 induce la

producción de fibronectina y colágenos (Weston et al., 2003, Chen et al., 2004). Además, CCN2 es capaz de regular la expresión de integrinas en la superficie celular, facilitando la acumulación de proteínas de matriz (Weston et al., 2003, Chen et al., 2004). Nuestro grupo ha utilizado el modelo de daño renal inducido por administración sistémica de Ang II en ratas para estudiar el papel de CCN2 en el inicio y progresión del daño renal *in vivo*. En este modelo se observó un rápido aumento en la expresión de CCN2 en el riñón al cabo de 3 días (células tubuloepiteliales y glomerulares) que se mantuvo elevada hasta los 15 días, tiempo en el que aparece fibrosis renal (Ruperez et al., 2003). Además, se observó que los niveles de TGF-β activo no aumentaron hasta las dos semanas (Carvajal et al., 2008), lo que indicó que CCN2 participa en el inicio del proceso fibrótico previo a las acciones profibróticas de TGF-β. En modelos experimentales de daño renal, como el de obstrucción unilateral del uréter, nefritis por anticuerpos anti-Thy1, glomeruloesclerosis diabética e infusión de Ang II se ha observado sobreexpresión de CCN2 asociado a fibrosis renal (Sánchez-López et al., 2009a, Hlubocka et al., 2002, Imai et al., 1992). Diversos estudios han sugerido que CCN2 podría ser una diana antifibrótica. Concretamente en la enfermedad renal experimental, el bloqueo de CCN2 mediante oligonucleótidos antisentido o por silenciamiento génico redujo la fibrosis túbulointersticial en la nefropatía obstructiva (Yokoi et al., 2004) y en modelos de diabetes por estreptozotocina (Guha et al., 2007).

Varios estudios sugieren la participación de CCN2 en la regulación del proceso inflamatorio. CCN2 regula adhesión celular y migración e induce, dependiendo del contexto celular, la expresión de mediadores pro-inflamatorios, como citoquinas y quimioquinas (Kular et al., 2011). Así, en cardiomiocitos induce la expresión de TNF-α, IL-6, IL8 y MCP-1 (Wang et al., 2010), en células pancreáticas aumenta los niveles de IL-1β e IL-6 (Karger et al., 2008), en células mesangiales incrementa MCP-1, RANTES y fractalquina (Wu et al., 2008) y en células tubulares MCP-1 y RANTES (Sanchez-López 2009b). Nuestro grupo ha demostrado que la administración de CCN2(IV) en ratón es capaz de inducir a las 24 horas una respuesta inflamatoria en el riñón, caracterizada por reclutamiento de células inflamatorias en el intersticio, producción de citoquinas proinflamatorias (IFN-γ, IL-6 y IL-4), y quimioquinas (MCP-1 y RANTES) mediante la activación del factor de transcripción NF-κB (Sánchez-López 2009b).

Diversos estudios han demostrado el papel crucial de las integrinas, de los proteoglicanos heparan sulfato (HSPGs) y del receptor de la proteína de baja densidad (LPR) en las respuestas celulares de CCN2 (Gao y Brigstock 2004, Gao y Brigstock 2003). La posibilidad de unión de CCN2 a receptores en la membrana celular fue sugerida en 1998 en estudios en condrocitos (Nishida et al 1998). Recientemente se ha descrito la existencia de un posible receptor para CCN2, el receptor tirosina quinasa del factor de crecimiento nervioso (TrkA) en células mesangiales (Wahab et al., 2005). Sin embargo, la existencia de un receptor específico no ha sido todavía descrita.

5.2. PROTEÍNA RELACIONADA CON LA PARATOHORMONA (PTHrP)

La PTHrP es una proteína que fue identificada en 1987 como responsable de la hipercalcemia tumoral de origen humoral (Esbrit 2001, Rankin et al., 1997). La sobreexpresión de la PTHrP es responsable del incremento tanto de la reabsorción ósea como de la reabsorción de calcio en el síndrome paraneoplásico (Philbrick et al., 1996). Esta proteína puede sufrir procesamiento postraduccional inducido por diversas enzimas, entre las que se encuentran las de tipo furina, generándose un fragmento N-terminal (1-36) de 36 aa, un fragmento medio (del aa 88 al 106) cuyo extremo C-terminal presenta dominios de localización nuclear y, por último, uno o varios fragmentos C-terminales que se generan a partir del aa 107 (Clemens et al., 2001, Philbrick et al., 1996). La PTHrP está expresada de forma constitutiva en muchos tejidos, y ejerce sus acciones a través de su interacción con el receptor PTH1R, de tipo GPCR

(Jüppner 1994), participando en procesos como la regulación del tono muscular liso, modulación del transporte de Ca^{2+} en el epitelio tubular y regulación del crecimiento, diferenciación, y apoptosis celular (Clemens et al., 2001, Henderson et al., 1995, Philbrick et al., 1996).

PTHrP participa en embriogénesis y desarrollo fetal. La supresión del gen de la PTHrP y del PTH1R es letal en ratones homocigotos, mientras que los heterocigotos son viables, pero presentan marcada osteopenia (Jüppner 1994, Strewler 2000). Durante el desarrollo embrionario renal se han observado niveles elevados de PTHrP en túbulo colector y glomérulos, pero no se detectó expresión del PTH1R en estas estructuras (Aya et al., 1999). En el riñón adulto PTHrP está presente en células mesangiales, podocitos, túbulos dístales, proximales y colectores (Lorenzo et al., 2002, Massfelder et al., 1996, Yang T et al., 1997)

Algunos estudios sugieren que la PTHrP podría tener un papel relevante en patología renal. La PTHrP está aumentada en diversas nefropatías experimentales, como la nefropatía isquémica por nefrotóxicos (Ortega et al., 2005, Santos et al., 2001, Soifer et al., 1993), modelos de obstrucción unilateral de uréter (Ramila et al., 2008), y daño renal mediado por Ang II (Lorenzo et al., 2002). Además, se ha observado que el tratamiento con bloqueantes de Ang II recupera la función renal en el fracaso renal agudo por nefrotóxicos y disminuye el componente inflamatorio en el riñón obstruido, asociado a una normalización en los niveles de PTHrP en el riñón (Ortega et al., 2005, Ramila et al., 2008). En células tubulares en cultivo, PTHrP induce TEM a través de su interacción con VEGF (Ardura et al., 2008), y regula la supervivencia modulando proteínas anti-apoptoticas, como Bcl-2 u osteopontina vía Runx2 (Ardura et al., 2013). Previamente se ha descrito que en células tubuloepiteliales y osteoblastos TGF-β podría modular algunas acciones de PTHrP mediadas por PTH1R (Law et al., 1993, Yang et al., 2007). En una línea celular de osteosarcoma humano la sobreexpresión de PTH1R aumentó su capacidad invasiva, asociada a la inducción de TGF-β (Yang et al., 2007). PTH induce la expresión de TGF-β y estimula la producción de colágeno tipo I en células osteoblásticas (Sowa et al., 2003, Wu et al., 2000), sin embargo no hay datos sobre PTHrP.

PTHrP vía PTH1R activa diversas vías de señalización incluidas PKA, PLC y PKC (Abou-Samra et al., 1992, Esbrit y Egido 2000), que posteriormente pueden confluir en la activación de la cascada de las MAPKs (Carpio et al., 2001, Miao et al., 2001). Sin embargo no hay datos sobre la vía del EGFR.

5.3. FAMILIA DE PROTEINAS TNFSF

La superfamilia de citoquinas estructuralmente relacionadas con el factor de necrosis tumoral (TNFSF), está considerada una fuente de dianas terapéuticas para el estudio de enfermedades humanas, y se ha descrito su implicación en gran variedad de respuestas biológicas (Wiley y Winkles 2003, Campbell et al., 2004, Baxter et al., 2006, Marsters et al., 1998, Foster et al., 2004). TNF-α es una citoquina pleiotrópica e inflamatoria que es sintetizada como una proteína de membrana en respuesta a inflamación, infección y daño (Baud y Karin 2001). TNF-α presenta un rol patogénico en un gran número de enfermedades inflamatorias crónicas, como la artritis reumatoide (Clark et al., 2007), participando también en la patología renal, tanto aguda como crónica (Vielhauer et al., 2007, Pascher y Klupp 2005).

Los ligandos de la familia de TNF se expresan originariamente como proteínas transmembrana de tipo II, pero en algunos casos son procesadas generando pequeñas proteínas secretables que presentan actividad biológica (Locksley et al., 2001, Bodmer et al., 2002). Ambas, las ancladas a membrana y las citoquinas solubles, contienen un

dominio C-terminal de homología con TNF que media en su trimerización y posterior unión a su receptor. Los miembros de la superfamilia de TNF se unen a uno o mas miembros de la superfamilia de receptores de TNF (TNFRSF) que está constituida en su mayoría por proteínas transmembrana tipo I o III (Locksley, et al., 2001, Bodmer, et al., 2002). Estos receptores están caracterizados por la presencia de un región extracelular de unión a ligando con varios dominios ricos en cisteínas y una cola citoplasmática con sitios de unión a proteínas adaptadoras.

5.3.1. *Citoquina inductora de apoptosis semejante a TNF* ***(TWEAK)***

TWEAK se sintetiza como una proteína transmembrana tipo II y, tras su procesamiento por la enzima furina, da lugar a una citoquina soluble biológicamente activa (Chicheportiche et al., 1997, Locksley et al., 2001, Bodmer et al., 2002). TWEAK es un ligando del receptor Fn14 (factor de crecimiento de fibroblastos inducible-14) (Wiley et al., 2001, Nakayama et al., 2003, Wiley et al., 2003, Brown et al., 2003, Han et al., 2003, Ruiz Ortega et al. 2014). Este receptor es una proteína transmembrana de tipo I, de 129 aa, que puede ser procesada dando lugar a una proteína madura de 102 aa, lo que la define como el miembro más pequeño de los TNFRSF (Wiley et al., 2001, Feng et al., 2000). La interacción de TWEAK con el monómero de Fn14, induce su trimerización y la posterior transducción de señales intracelulares (Meighan-Mantha et al., 2009, Campbell et al., 2004, Justo et al., 2006, Winkles 2008). La cola citoplasmática de Fn14 no presenta el dominio clásico de muerte TNFR, pero activa numerosas cascadas de señalización intracelular, incluyendo NF-κB y la cascada de las MAPKs a través de su interacción con las moléculas adaptadoras TRAFs (*TNF-associated factors*) (Wiley et al., 2001, Locksley et al., 2001, Inoue Jet al., 2000, Wajant et al., 1999) **(Figura 7)**. Recientemente se ha descrito que en monocitos, TWEAK también podría unirse a CD163, un *receptor scavenger* rico en cisteínas (Bover et al., 2007) **(Figura 7)**. Esta interacción TWEAK/CD163 puede tener relevancia biológica durante la respuesta inflamatoria y en procesos tumorales, pero hasta el momento Fn14 es conocido como el único receptor de TWEAK inductor de respuestas intracelulares.

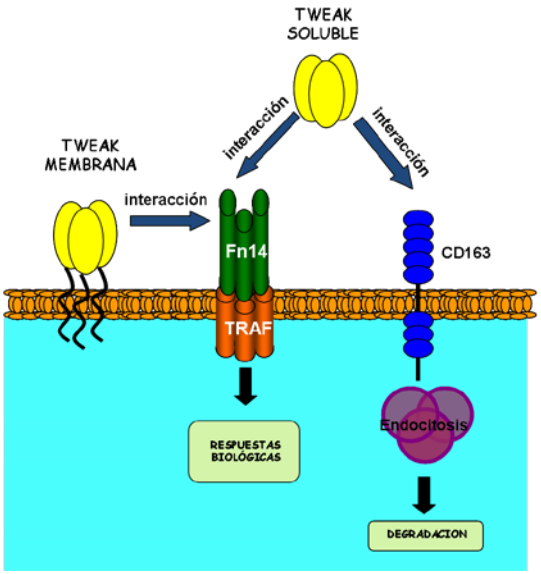

Figura 7: Receptores descritos para TWEAK: Fn14 (receptor inductor de respuestas biológicas) y CD163 (receptor *scavenger*).

TWEAK regula una gran variedad de respuestas celulares, incluidas el crecimiento celular/apoptosis, la angiogénesis y la inflamación (Locksley et al., 2001). De entre todas, TWEAK regula diferentes moléculas implicadas en la respuesta inflamatoria. Los primeros estudios se realizaron en fibroblastos dérmicos humanos donde se describió que TWEAK aumentaba la producción de las moléculas proinflamatorias PGE2, IL-6, IL-8, RANTES e IP-10 (Chicheportiche et al., 2002), estudios que han sido confirmados en otros tipos celulares, incluidos células mesangiales (Kim et al., 2004) y células túbulo epiteliales (Sanz et al., 2008). La administración sistémica de TWEAK a ratones induce una respuesta inflamatoria en el túbulointersticio renal, caracterizada por un aumento de la expresión génica de MCP-1, RANTES, IL-6 y CCR2 (receptor de MCP-1), y de la presencia de macrófagos (Sanz et al., 2008).

TWEAK y su receptor Fn14 están presentes en muchos tejidos, incluido riñón adulto sano (Sanz et al., 2008). La expresión excesiva del sistema TWEAK/Fn14 conlleva respuestas patológicas en los tejidos, permitiendo la progresión del daño y la degeneración del tejido (Sanz et al., 2014, Burkly et al., 2011). Así, se ha descrito aumento de TWEAK/Fn14 a nivel de RNAm y proteína en el daño renal agudo, en aterosclerosis y otros desórdenes (Winkles 2008, Sanz et al., 2014, Blanco-Colio et al., 2011, Sanz et al.,2008, Sanz et al., 2010, Moreno et al., 2011). En ratones *knockout* para TWEAK donde se realizó un modelo de obstrucción unilateral del uréter se observó menor daño tubular temprano, infiltrado inflamatorio y fibrosis renal que los ratones de fenotipo salvaje (Ucero et al., 2013). Por otro lado, el bloqueo de TWEAK/Fn14 disminuyó el daño renal causado por acido fólico (Sanz et al., 2008) así como por isquemia/reperfusión (Hotta et al., 2011) en ratones. En ratones hiperlipidémicos APOE-/- con daño renal crónico no inmune, la administración de TWEAK activó la vía de señalización de NF-κB y aumentó la severidad del daño túbulointesticial y glomerular así como la inflamación, efecto que fue inhibido con el tratamiento con un anticuerpo neutralizante frente a TWEAK (Muñoz-García et al., 2009). En biopsias de pacientes con ERC se han detectado niveles elevados de TWEAK y Fn14 (Izquierdo et al., 2012, Park et al., 2012). Sin embargo, los niveles de TWEAK soluble circulante están disminuidos (Yilmaz et al., 2009, Ruiz Ortega et al., 2014), es decir TWEAK es un biomarcador inverso de daño.

TWEAK puede desempeñar distintas funciones dependiendo del tejido. En un modelo de daño peritoneal se ha observado que Fn14 y TWEAK activan las células mesoteliales y promueven la expresión de quimioquinas dependientes de la activación de NF-κB, como MCP1, con la finalidad de reclutar macrófagos a la cavidad peritoneal y al tejido (Sanz et al., 2014). En hígado, estudios en ratones que sobreexpresan TWEAK (transgénicos, o infectados con adenovirus) y ratones *Knockout* para Fn14, han demostrado el papel beneficioso de TWEAK en el proceso de regeneración hepática ya que TWEAK contribuye a la expansión de células progenitoras hepáticas después de un daño químico (Jakubowski, et al., 2005). TWEAK puede actuar como un regulador positivo de la miogénesis *in vivo* como ocurre en ratones *Knockout* para Fn14, que presentan una respuesta inflamatoria reducida y un retraso en la regeneración de las fibras musculares (Girgenrath, et al., 2006). Pero también puede actuar como regulador negativo de la musculatura, ya que la administración de TWEAK a ratones reduce el diámetro de las fibras musculares y el peso corporal (Dogra, et al., 2007). TWEAK también regula procesos de angiogénesis. En ratas, la inyección de TWEAK en la cornea causa una elevada actividad angiogénica, comparable con la de factores pro-angiogénicos ya descritos (Lynch et al., 1999). También TWEAK tiene un papel angiogénico destacable en procesos tumorales, en los que puede actuar de forma sinérgica con VEGF y FGF2 (Donohue et al., 2003, Jakubowski et al., 2002).

6. FÁRMACOS PARA EL TRATAMIENTO DE LA ENFERMEDAD RENAL

6.1. ANÁLOGOS DE LA VITAMINA D: PARICALCITOL

Las hormonas esteroideas actúan como mensajeros químicos en múltiples tejidos, entre ellas destaca la vitamina D3, que se produce en la piel a partir del esteroide 7-dehidrocolesterol por irradiación ultravioleta (Bringhurst et al., 2006). La vitamina D está unida a la proteína transportadora DBP, con la que viaja por el flujo sanguíneo hasta el hígado. Allí se hidroxila transformándose en 25(OH)-vitamina D (calcidiol) que es la forma circulante primaria de vitamina D y la que suele medirse en sangre. Posteriormente, en los riñones, se hidroxila a 1,25-dihidroxivitamina D (calcitriol), que es la forma biológicamente activa de la vitamina D (Bringhurst et al., 2006) **(Figura 8).**

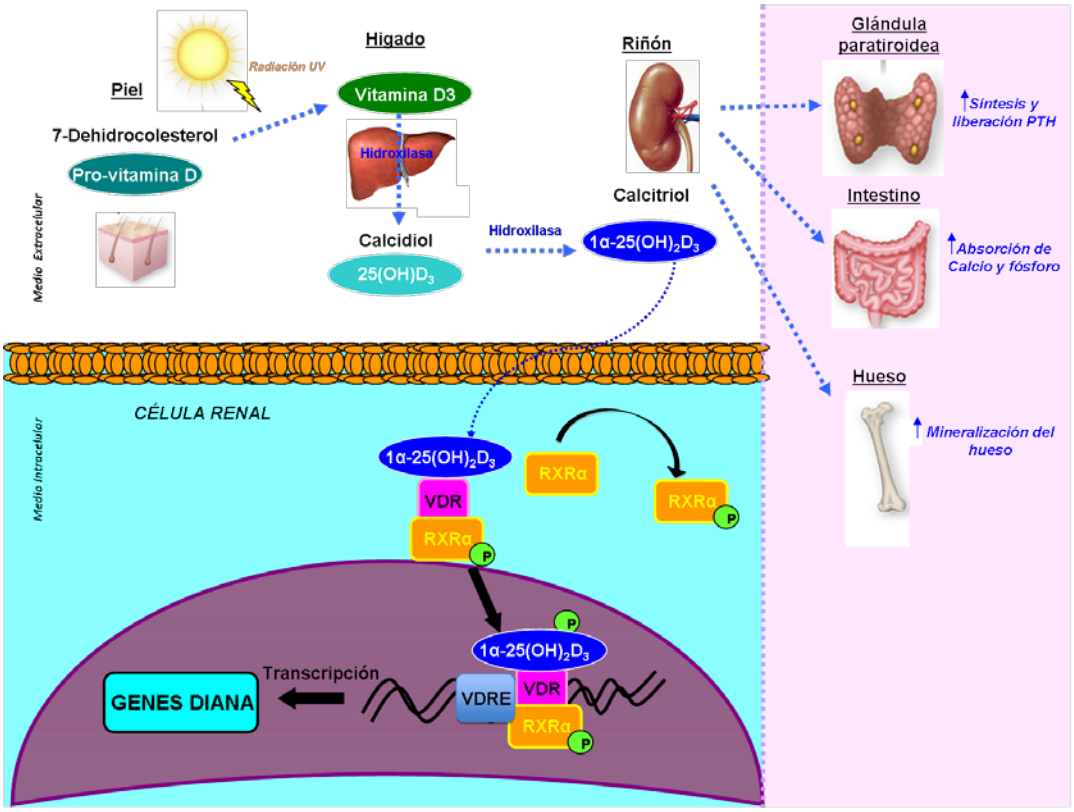

Figura 8: Proceso de síntesis de la Vitamina D y su señalización intracelular en la célula renal. Efectos de la vitamina D en otros tejidos.

El calcitriol ejerce sus efectos a través de la interacción con su receptor específico, el receptor de la vitamina D (VDR). El VDR es un miembro de una superfamilia de receptores nucleares entre los que se encuentran los receptores de hormonas tiroídeas, glucocorticoides, estrógenos, andrógenos, ácido retinoico (RXR) y receptores activadores de la producción de peroxisomas (PPAR) (Evans 1988, Pike 1992). Se ha descrito que VDR presenta una mayor actividad transcripcional cuando forma un complejo heterodimérico con RXR (Kraichely y MacDonald 1998). El calcitriol se une al complejo VDR-RXR, y se trasloca al núcleo, donde se une a los elementos de respuesta a VDR (VDRE) y actúa como un factor de transcripción regulando la expresión de sus genes diana (Jurutka et al.,2001, MacDonald et al., 2001, Rachez et al., 2000) **(Figura 8).** Los efectos del calcitriol modulados por la unión a VDR son

los denominados efectos de respuesta genómica. En ocasiones, calcitriol media efectos rápidos e independientes de VDR, que se conocen como efectos de respuesta no genómica. (Barsony y Marx 1988)

El Calcitriol se caracteriza por ser un factor clave en la homeostasis mineral (Pike 1992). Sin embargo, múltiples estudios sugieren un papel distinto, más allá de su efecto sobre el metabolismo mineral y óseo, presentado efectos beneficiosos en patologías diversas, entre las que se incluyen enfermedades autoinmunes (Evans 1988, Arnson et al., 2007, Cantorna 2000, DeLuca y Cantorna 2001), inhibiendo la proliferación en procesos cancerosos (Haussler 1986, Holick 2004, Giovannucci 2005), o regulando la diferenciación celular en psoriasis (Holick 1993, Kragballe 1992).

Una de las mayores complicaciones en los pacientes con ERC es el desarrollo de hiperparatiroidismo secundario. En el pasado el incremento de la síntesis de la hormona paratiroidea (PTH) estaba atribuido a un descenso en los niveles de calcio, y por ello, para corregir esta hipocalcemia, se trataba a los enfermos con metabolitos de la vitamina D. La combinación de un aumento en los niveles de PTH y una disminución en niveles de calcitriol se asocia a pérdida ósea, enfermedad cardiovascular, supresión inmunológica y aumento de mortalidad en pacientes en estadio final de ERC (Andress 2006). De hecho, existe una correlación positiva entre el descenso de los niveles de calcitriol y la pérdida de la función renal (Martinez et al., 1996). Por ello, el uso de los agonistas del receptor de la vitamina D (VDRAs) **(Figura 9)** se ha convertido en un tratamiento muy extendido en la práctica clínica, sobretodo en los pacientes con ERC en diálisis (Andress 2006).

Aunque el uso de VDRAs está asociado a la supervivencia (Kalantar-Zadeh et al., 2006, Marco et al., 2001, Melamed ML et al. 2006, Naves-Diaz et al., 2008, Shoji et al., 2004, Teng et al., 2005, Tentori et al., 2006, Wolf et al., 2007), existen ciertos problemas con su aplicación en clínica ya que este tratamiento induce un incremento en los niveles de calcio y fósforo, hecho relacionado con un elevado riesgo de mortalidad en pacientes en tratamiento con diálisis; lo que hace que sea cada vez mas necesario el uso de VDRAs menos calcémicos y fosfatémicos sobre todo en enfermos con ERC. Uno de los primeros estudios en el que se comparó calcitriol *vs* paricalcitol en pacientes con ERC fue realizado por Teng *y cols*, donde se observó como paricalcitol mejoraba la tasa de supervivencia (Teng et al., 2003). Estudios clínicos posteriores han descrito que este análogo presenta una reducción mas rápida de los niveles de PTH y menos episodios calcémicos comparado con calcitriol (Sprague et al., 2003).

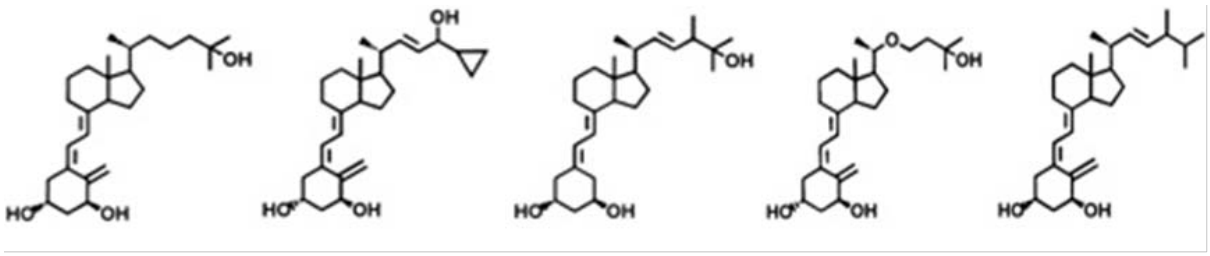

Figura 9: Análogos de la vitamina D.

Múltiples estudios han demostrado que el uso de VDRAs, como paricalcitol, en enfermos con ERC reduce los niveles de proteinuria independientemente de la tasa de filtración glomerular (Agarwal et al., 2005), disminuye la

excreción de albúmina (Kuhlmann et al., 2004), retarda la progresión de glomeruloesclerosis (Schwarz et al., 1998), mejora la supervivencia y reduce la incidencia de enfermedad cardiovascular (Valdivielso et al., 2009, Levin et al., 2007, Perez-Gomez et al., 2013). En la enfermedad renal experimental el tratamiento con la vitamina D o con VDRAs disminuye la fibrosis, proliferación mesangial y perdida podocitaria (Valdivielso et al., 2009, Levin et al., 2007, Perez-Gomez et al., 2013, González-Parra et al., 2012, Bodyak et al., 2007). Estudios recientes se han centrado en el papel de los VDRAs en enfermedades renales glomerulares primarias (Hirata et al., 2002, Kuhlmann et al., 2004, Panichi et al., 2001). Se ha observado que la forma activa de la vitamina D presenta efectos beneficiosos en la nefropatia obstructiva, modelo caracterizado por una infiltración inflamatoria, atrofia tubular y fibrosis (Tan et al., 2006). Ratones *knockout* para VDR desarrollan hipertensión con niveles elevados de renina, hipertrofia cardiaca, y una susceptibilidad al daño tras una obstrucción uréteral (Li et al., 2002, Xiong et al., 2012). Los efectos terapéuticos de la vitamina D en la nefropatia obstructiva están asociados a su capacidad de preservar la integridad el túbuloepitelio inhibiendo la TEM (Tan et al., 2006). Sin embargo, observando sus propiedades pleiotrópicas se puede pensar que los efectos renoprotectores de la vitamina D pueden deberse a múltiples acciones (Tian et al., 2007, Andress et al., 2006). Sin embargo, los mecanismos a través de los cuales los VDRAS desempeñan sus efectos antinflamatorios en daño renal no se conocen.

II. OBJETIVOS

OBJETIVO GENERAL

Independientemente de la etiología subyacente, muchas enfermedades renales crónicas (ERC) tienen en común un proceso inflamatorio que desemboca en una fibrosis progresiva contribuyendo al fallo renal terminal. Los actuales tratamientos sólo consiguen frenar la progresión de la enfermedad renal progresiva. El objetivo general de esta tesis se centra en evaluar la contribución de vía del EGFR en daño renal experimental, con el fin de mejorar las actuales estrategias terapéuticas empleadas en los pacientes con ECR.

Estudios experimentales sugieren que la transactivación del EGFR por factores sobreexpresados en daño renal podría ser un mecanismo clave en la fibrosis, pero no hay datos sobre su papel en la modulación de la respuesta inflamatoria. En esta tesis evaluaremos si diversos factores implicados en el daño renal, como son CCN2, PTHrP y TWEAK, son capaces de activar la ruta del EGFR, investigando los mecanismos moleculares y celulares implicados en sus respuestas renales (con especial atención a la fibrosis e inflamación). Entre los actuales tratamientos en ECR los agonistas del receptor de la vitamina D (VDRAs) presentan efectos renoprotectores. Por último, investigaremos si la ruta EGFR participa en los efectos antinflamatorios producidos por estos fármacos.

Objetivos específicos

1. CCN2 es un mediador de las acciones profibróticas de AngII y TGF-β. La transactivación del EGFR por AngII se ha descrito como un proceso clave en la fibrosis renal experimental. Recientemente nuestro grupo ha descrito que CCN2 es una citoquina proinflamatoria que regula la inflamación renal. Se investigará si CCN2 activa la ruta del EGFR en riñón y los mecanismos moleculares y procesos celulares asociados (inflamación y fibrosis). Para ello, se realizarán estudios en células renales en cultivo y en un modelo experimental de administración sistémica de CCN2 en ratón.
2. PTHrP se ha implicado en procesos de proliferación y fibrosis renal. Se investigará si la PTHrP activa la ruta del EGFR en riñón y su contribución a la TEM renal. Para ello, se realizarán estudios en células tubuloepiteliales renales en cultivo y en un modelo experimental de ratón de sobreexpresión de PTHrP específica en el túbuloepitelio renal .
3. TWEAK es un citoquina proinflamatoria muy relevante en la patología renal. Se investigará si TWEAK activa la ruta del EGFR en riñón, los mecanismos intracelulares (papel de ADAMs y ligandos del EGFR; TGF-α, HB-EGF) y su contribución a la inflamación renal. Para ello, se realizarán estudios en células tubuloepiteliales renales en cultivo y en un modelo experimental de administración sistémica de TWEAK en ratón.
4. Se evaluará si la ruta EGFR está modulada por fármacos empleados en clínica. En concreto, investigaremos si esa ruta está asociada a los efectos beneficiosos de los VDRAs. Para ello se estudiará el efecto del tratamiento con el VDRA Paricalcitol en un modelo de inflamación renal inducido por la administración sistémica de TWEAK en ratón.

III. MÉTODOS Y RESULTADOS

1. <u>CCN2 es un nuevo ligando del receptor del factor de crecimiento epidérmico (EGFR) y regula la respuesta inflamatoria renal.</u>

CCN2 es un gen de desarrollo que no está presente en el riñón adulto y se reexpresa en situaciones de daño renal. Además, se ha sugerido que podría ser una diana terapéutica y un biomarcador de daño en la ERC. Inicialmente CCN2 fue considerado como un factor profibrótico. Sin embargo, los estudios realizados en los últimos años han ampliado esta idea y ha pasado a considerarse como un factor multifuncional implicado en distintos procesos como la regulación de la proliferación/apoptosis, la angiogénesis, la migración, la adhesión y, más recientemente, la inflamación (Lau y Lam 1999). Sin embargo, su receptor específico no se conoce. Cada vez existen más estudios que sugieren que la modulación de la activación del EGFR podría ser una diana terapéutica para la ERC. La expresión del EGFR se ha localizado en fibroblastos (Stevens et al., 2007) y en células epiteliales renales (Wu et al., 2009) pero la posibilidad de que CCN2 active al EGFR se desconoce.

En esta parte de la tesis se aborda el objetivo 1 Los resultados obtenidos muestran como la molécula completa de CCN2 y su fragmento C-terminal (CCN2(IV)) son capaces de activar el EGFR en células tubuloepiteliales en cultivo. Además, el bloqueo farmacológico y génico de MMPs y en concreto de ADAM17, no revirtió la activación del EGFR, lo que descartó una posible transactivación del EGFR inducida por CCN2(IV). Mediante la técnica de resonancia de plasmón superficial (RPS) se observó que CCN2 podría ser un nuevo ligando del EGFR. Además, CCN2 y su fragmento C-terminal muestran una clara interacción con la forma soluble del EGFR, sugiriendo que el sitio de unión a EGFR está localizado en este modulo de CCN2. *In Vivo* la administración sistémica de CCN2(IV) indujo la activación de la vía del EGFR en riñón, localizada concretamente en las células tubuloepiteliales. Para demostrar la implicación de la vía del EGFR en las respuestas renales inducidas por CCN2, se bloqueó *in vivo* la quinasa del EGFR mediante un inhibidor farmacológico (Erlotinib) observando una disminución en los cambios renales inducidos por CCN2(IV). *In Vitro* se ha demostrado que ambas moléculas, CCN2 y CCN2(IV) activan la vía del EGFR mediante la interacción física con EGFR (estudios de inmunoprecipitación) e inducen su fosforilación y posterior activación de cascadas de señalización intracelulares como la vía de la quinasa ERK y la inducción de factores proinflamatorios. Previamente se ha descrito que CCN2 interacciona con el receptor de neurotrofina TrKA en células mesangiales (Wahab et al., 2005) y que, además, existe una interrelación entre las vías de señalización activadas por ambos receptores EGFR/TrkA en monocitos (El Zein et al., 2010). *In vitro* se observó que CCN2(IV) también es capaz de activar TrKA. El bloqueo farmacológico y génico, tanto de TrKA como de EGFR, inhibió la fosforilación del EGFR y del TrKA inducida por CCN2(IV), respectivamente. Estos resultados demuestran la existencia de un posible *crosstalk* entre los receptores EGFR/TrkA en respuesta a la estimulación con CCN2(IV). Además, el bloqueo del CCN2 endógeno inhibió la transactivación del EGFR inducida por TGF-β, lo que sugiere el papel que ejerce CCN2 como mediador sobre las acciones de TGF-β. Estos resultados sugieren que CCN2 podría ser un nuevo ligando del EGFR que participa en el daño renal través de la vía de señalización del EGFR.

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Article

Connective tissue growth factor is a new ligand of epidermal growth factor receptor

Sandra Rayego-Mateos[1], Raquel Rodrigues-Díez[1], Jose Luis Morgado-Pascual[1], Raul R. Rodrigues Díez[1], Sebastian Mas[3], Carolina Lavoz[1], Matilde Alique[1], Janos Pato[2], Gyorgy Keri[2,3], Alberto Ortiz[4], Jesus Egido[4], and Marta Ruiz-Ortega[1,*]

[1] Cellular Biology in Renal Diseases Laboratory, Universidad Autónoma de Madrid, 28040 Madrid, Spain
[2] Pathobiochemistry Research Group of Hungarian Academy of Sciences, Semmelweis University, H-1094 Budapest, Hungary
[3] Vichem Chemie Research Ltd., H-1022 Budapest, Hungary
[4] Renal Research Laboratory, IIS-Fundación Jiménez Díaz, Universidad Autónoma de Madrid, 28040 Madrid, Spain
* Correspondence to: Marta Ruiz-Ortega, E-mail:mruizo@fjd.es

Chronic kidney disease is reaching epidemic proportions worldwide and there is no effective treatment. Connective tissue growth factor (CCN2) has been suggested as a risk biomarker and a potential therapeutic target for renal diseases, but its specific receptor has not been identified. Epidermal growth factor receptor (EGFR) participates in kidney damage, but whether CCN2 activates the EGFR pathway is unknown. Here, we show that CCN2 is a novel EGFR ligand. CCN2 binding to EGFR extracellular domain was demonstrated by surface plasmon resonance. CCN2 contains four distinct structural modules. The carboxyl-terminal module (CCN2(IV)) showed a clear interaction with soluble EGFR, suggesting that EGFR-binding site is located in this module. Injection of CCN2(IV) in mice increased EGFR phosphorylation in the kidney, mainly in tubular epithelial cells. EGFR kinase inhibition decreased CCN2(IV)-induced renal changes (ERK activation and inflammation). Studies in cultured tubular epithelial cells showed that CCN2(IV) binds to EGFR leading to ERK activation and proinflammatory factors overexpression. CCN2 interacts with the neurotrophin receptor TrkA, and EGFR/TrkA receptor crosstalk was found in response to CCN2(IV) stimulation. Moreover, endogenous CCN2 blockade inhibited TGF-β-induced EGFR activation. These findings indicate that CCN2 is a novel EGFR ligand that contributes to renal damage through EGFR signalling.

Keywords: CCN2, receptors, EGFR, TrkA, renal, inflammation

Introduction

Chronic kidney disease is a major health problem that has reached epidemic proportions and it may lead to end-stage renal disease or early cardiovascular death. Moreover, available clinical treatments only retard renal disease progression. Connective tissue growth factor (CCN2/CTGF), a member of the CCN (Cyr61/CCN2/Nov) family, is over-expressed in many human renal pathologies (Perbal, 2004; De Winter et al., 2008). Experimental studies have shown that CCN2 inhibition slows disease progression in diabetic nephropathy, unilateral ureteral obstruction, and nephrectomized TGF-β1 transgenic mice (Yokoi et al., 2004; Okada et al., 2005; Guha et al., 2007; Phanish et al., 2010) suggesting that therapeutic approaches that selectively block CCN2 activity could be beneficial for renal disease treatment.

CCN2 has to be considered a matricellular protein rather than a conventional growth factor. This protein, as other CNN members, contains four distinct structural modules that can be cleaved by proteases: an amino-terminal insulin-like growth-factor-binding domain, a cysteine-rich domain, a thrombospondin type 1 repeat, and a carboxyl-terminal cystine-knot domain (Rachfal and Brigstock, 2005; Leask and Abraham, 2006; De Winter et al., 2008; Chen and Lau, 2010). CCN2 and its degradation fragments have been detected in biological fluids and have been proposed as risk biomarkers in several nephropathies (Riser et al., 2003; Tam et al., 2009; Slagman et al., 2011). Among these degradation fragments, the 11 kDa carboxyl-terminal module (namely here CCN2(IV)) has received special interest. In cultured cells, this fragment regulates cell migration and proliferation, increases chemokines and extracellular matrix production, and has been involved in renal inflammation (Liu et al., 2006; De Winter et al., 2008; Sanchez-Lopez et al., 2009; Markiewicz et al., 2011). Although several studies have investigated the intracellular mechanisms activated by CCN2 and its fragments, the identification of a specific receptor for CCN2 remains elusive.

The epidermal growth factor receptor (EGFR) is the founding member of the ErbB receptor tyrosine kinase family. EGFR signalling controls key cellular programmes, including survival, proliferation, differentiation, and locomotion, both during development and postnatally. The EGFR is over-expressed, dysregulated, or mutated in many epithelial malignancies, participating in human cancer including lung, colon, breast, ovary, and gliomas (Sibilia et al., 2007; Bronte

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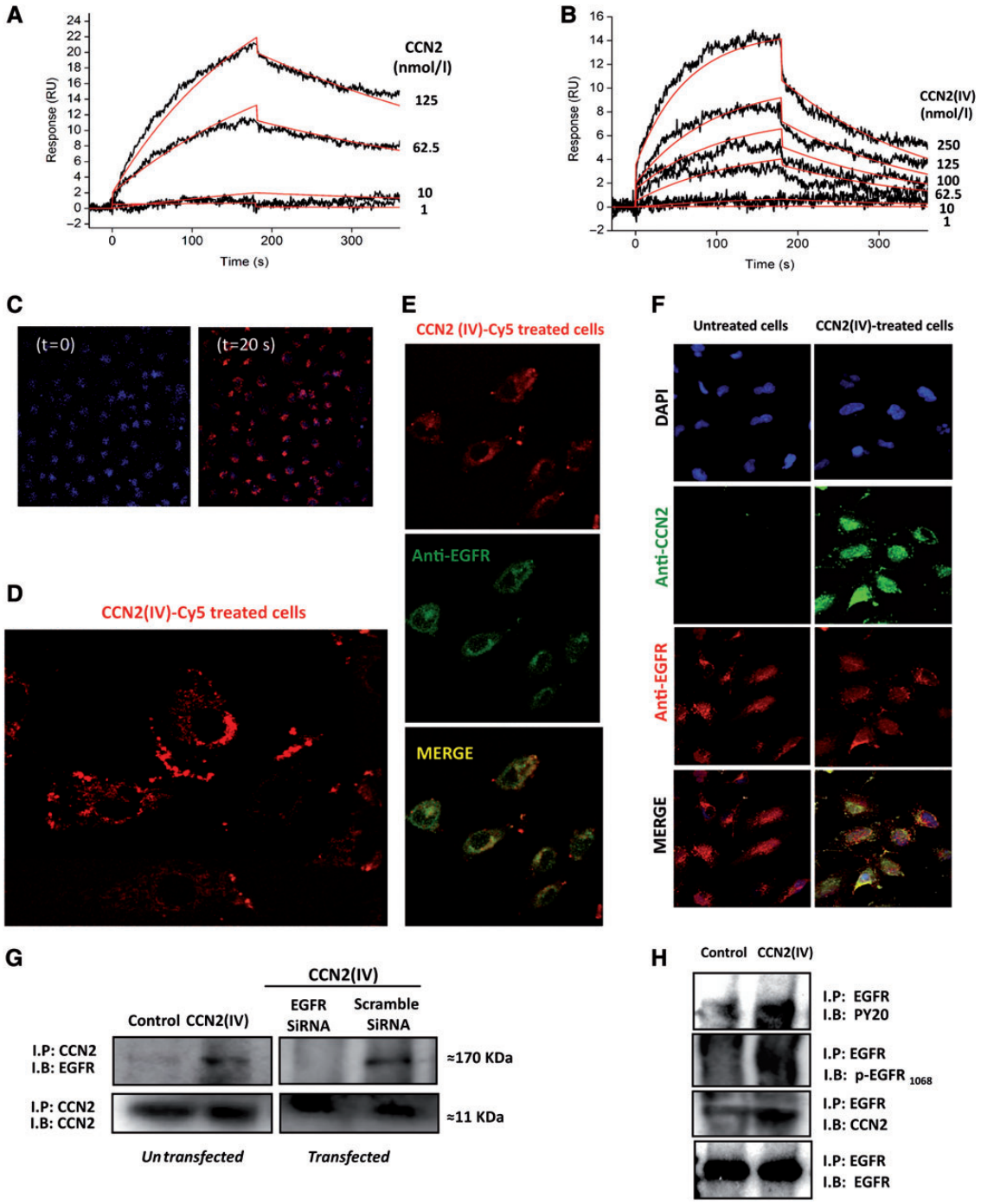

Figure 1 CCN2 binds to EGFR. Surface plasmon resonance interaction analysis of full-length CCN2 (**A**) and the carboxyl-terminal CCN2 fragment (CCN2(IV)) (**B**) with immobilized EGFR extracellular domain (sEGFR) was performed using Biacore 3000. Increasing concentrations of ligands (1–250 nmol/L) were injected over a surface with a density of 500 response units (RU) of immobilized sEGFR. The response in RU was recorded as a function of time. An overlay plot is shown of all sensorgrams after subtraction of their respective control sensorgrams. Binding parameters, calculated by applying the nonlinear curve-fitting software package BIAevaluation 3.2 (BIAcore, Inc.) to all sensorgrams simultaneously using a single-site model with drifting baseline, indicate that CCN2(IV)/EGFR interaction occurs with Kd $= 126 \pm 2$ nmol/L. (**C** and **D**) CCN2(IV) interacts with EGFR in human tubular epithelial cells. Fluorescent labelled CCN2(IV)-Cy5 (100 ng/ml) was added to HK2 cells and live confocal microscopy images were taken once every 1.3 sec for a period of 2 min (time 0 and 20 sec, **C**; time 2 min, **D**). Nuclei were stained with DAPI (blue). CCN2–EGFR interaction was evaluated by immunocytochemistry. Serum-starved HK2 cells were stimulated or not with 100 ng/ml CCN2(IV)-Cy5 (**E**) or CCN2(IV) (**F**) for 10 min, and fixed by cross-linking. (**E**) CCN2(IV)-Cy5-treated cells presented a red membrane immunostaining, while EGFR was immunodetected by an secondary AlexaFluor®488 labelled antibody (green). EGFR/CCN2(IV) colocalization was found (yellow staining, merge). (**F**) EGFR and CCN2 were detected using specific primary antibodies followed by their corresponding secondary AlexaFluor®633/488 antibodies (red/green, respectively). EGFR/CCN2 colocalization was found only in CCN2(IV)-treated cells (yellow staining), but not in control ones. Figures show a

et al., 2011). Besides tumour biology, EGFR family members are implicated in the development of end organ damage in hypertension (Hao et al., 2004) and atherosclerosis (Dreux et al., 2006). In the kidney, EGFR signalling is critically involved in renal electrolyte homeostasis (Melenhorst et al., 2008), and EGFR blockade, by genetic or pharmacological approaches, ameliorates experimental renal disease progression (Terzi et al., 2000; Lautrette et al., 2005; Flamant et al., 2012; Liu et al., 2012). Our aim was to investigate the capacity of CCN2 and its carboxyl-terminal fragment to interact with and activate EGFR, and whether the activation of EGFR signalling is involved in CCN2-induced responses in the kidney.

Results

CCN2 binds to EGFR via the carboxyl-terminal module

Surface plasmon resonance analysis was used to assess the ability of CCN2 to bind the extracellular domain of EGFR. CCN2 bound to immobilized EGFR on a BIAcore sensor chip (Figure 1A). The full-length CCN2 protein contains four distinct structural modules (De Winter et al., 2008). Binding experiments using the carboxyl-terminal degradation fragment CCN2(IV) as a ligand showed a clear interaction with EGFR (Figure 1B), suggesting that the EGFR-binding site is present in the carboxyl-terminal module.

To investigate whether CCN2 directly interacts with EGFR in cells, we performed studies in cultured human tubular epithelial cells (HK2 cell line). First, live-cell imaging by confocal time-lapse microscopy was performed to visualize CCN2(IV) binding to the cell. After adding labelled CCN2(IV)-Cy5 to cells, the immunofluorescent signal was rapidly located at the cell membrane, indicating CCN2(IV) cellular binding (Figure 1C and D). The potential CCN2–EGFR interaction was further demonstrated by immunocytochemistry and immunoprecipitation (IP) experiments, using a cross-linking procedure to fix the proteins anchored to the cell surface. EGFR is expressed in untreated HK2 cells. Moreover, CCN2(IV)-treated cells showed a clear cellular binding that colocalized with EGFR immunostaining (Figure 1E and F). IP studies showed that in CCN2(IV)-treated cells, but not in untreated ones, CCN2–EGFR complexes were formed (Figure 1G and H). One of the earliest steps of EGFR activation is its auto-phosphorylation on tyrosine (Y) residues (Sweeney and Carraway, 2000). In CCN2(IV)-treated cells, complexes containing tyrosine-phosphorylated proteins, including Y1068 on EGFR, were found (Figure 1H). Transfection with a small interfering RNA molecule (siRNA) targeting EGFR, but not with a nonspecific scramble siRNA, abolished the CCN2–EGFR complex formation, showing the specificity of this interaction (Figure 1G). These data demonstrate that in cultured tubular epithelial cells, stimulation with CCN2(IV) led to CCN2–EGFR complex formation.

CCN2 induces EGFR phosphorylation in cultured tubular epithelial cells

In cultured human tubular epithelial cells, CCN2(IV) increased EGFR phosphorylation on Y1068 and Y1173 (Figure 2A). In murine tubular epithelial cells, CCN2 (IV)-induced EGFR activation was dose- and time-dependent, starting as early as 5 min and peaking after 15 min with a maximal response at 50 ng/ml (Figure 2B and C). EGFR specific activation was demonstrated by pharmacological inhibition using two different EGFR kinase inhibitors, erlotinib and AG1478 (Figure 2D), and EGFR gene silencing (Figure 2E). CCN2(IV) also increased EGFR phosphorylation in other cell types, including murine fibroblasts and human mesangial cells (Figure 2F and G). Moreover, the full-length CCN2 protein also induced EGFR phosphorylation (Figure 2H), showing a similar response as obtained with the carboxyl-terminal fragment CCN2(IV). For this reason, only CCN2(IV) was used in the following experiments. Our data demonstrate that both CCN2 and its carboxyl-terminal fragment bind and activate EGFR signalling in cells.

CCN2(IV) induces EGFR phosphorylation in the kidney

Next, we investigated whether CCN2 could activate EGFR signalling in the kidney. Renal levels of phosphorylated EGFR protein were elevated in CCN2(IV)-injected mice compared with control mice (Figure 3A). In the kidney, EGFR is mainly expressed in tubular cells (Melenhorst et al., 2008). Immunohistochemistry and immunofluorescence using antibodies that recognized phosphorylated EGFR on Y1173 and Y1068, respectively, revealed that CCN2(IV) activated EGFR in tubular cells *in vivo* (Figure 3B, C, and E). Treating the CCN2(IV)-injected mice with erlotinib, a small molecule tyrosine kinase inhibitor that targets the receptor catalytic domain of EGFR, diminished renal phosphorylated EGFR levels to control levels (Figure 3B, C, and E).

EGFR activation by CCN2(IV) is linked to ERK signalling

Several auto-phosphorylation sites have been identified in the carboxyl-terminal region of EGFR that varies dependent on the ligand and are linked to different downstream signalling systems (Sweeney and Carraway, 2000). EGFR phosphorylation on Y1068 and Y1173 is involved in ERK signalling (Rojas et al., 1996; Pourazar et al., 2008). Both tyrosines were phosphorylated in kidneys of CCN2(IV)-injected mice (Figure 3) and in cultured tubular epithelial cells exposed to CCN2(IV) (Figure 2A). CCN2(IV)-injected mice also presented elevated renal levels of phosphorylated ERK1/2 compared with controls, which were inhibited by erlotinib (Figure 3D). EGFR activation by CCN2 was also linked to ERK signalling in cultured tubular epithelial cells. Blockade of EGFR, by kinase inhibition or gene silencing, diminished ERK phosphorylation levels in CCN2(IV)-treated cells to levels similar to their corresponding controls (Figure 2D and E).

CCN2(IV) via EGFR activation regulates renal inflammatory response in vivo and in vitro

We further investigated the *in vivo* effect of EGFR blockade on CCN2-induced renal damage. Treatment of CCN2(IV)-injected mice with erlotinib diminished the presence of infiltrating monocytes/macrophages ($F4/80^+$ cells) and T lymphocytes ($CD3^+$

representative experiment of four with similar results. (**G**) CCN2–EGFR complexes were found by coprecipitation experiments. Cell lysates were immunoprecipitated with anti-CCN2, followed by SDS–PAGE and western blotting (IB) using an anti-EGFR antibody. In some points, cells were transfected with an EGFR siRNA or its corresponding scramble siRNA. In CCN2(IV)-treated cells, the 170 kDa band corresponding to EGFR molecular weight was found, while it disappeared in EGFR silenced cells, showing the formation of CCN2–EGFR complexes. The IB with anti-CCN2 antibody was used as loading control. (**H**) IP with anti-EGFR antibody followed by IB with several antibodies: anti-phosphorylated tyrosine (PY20), anti-phosphorylated EGFR ($p\text{-}EGFR_{1068}$), anti-CCN2, and anti-EGFR (used as loading control). In CCN2(IV)-treated cells, bands for PY20, $p\text{-}EGFR_{1068}$ and CCN2 were detected, confirming CCN2–EGFR complex formation. Figures show a representative IP experiment of five with similar results.

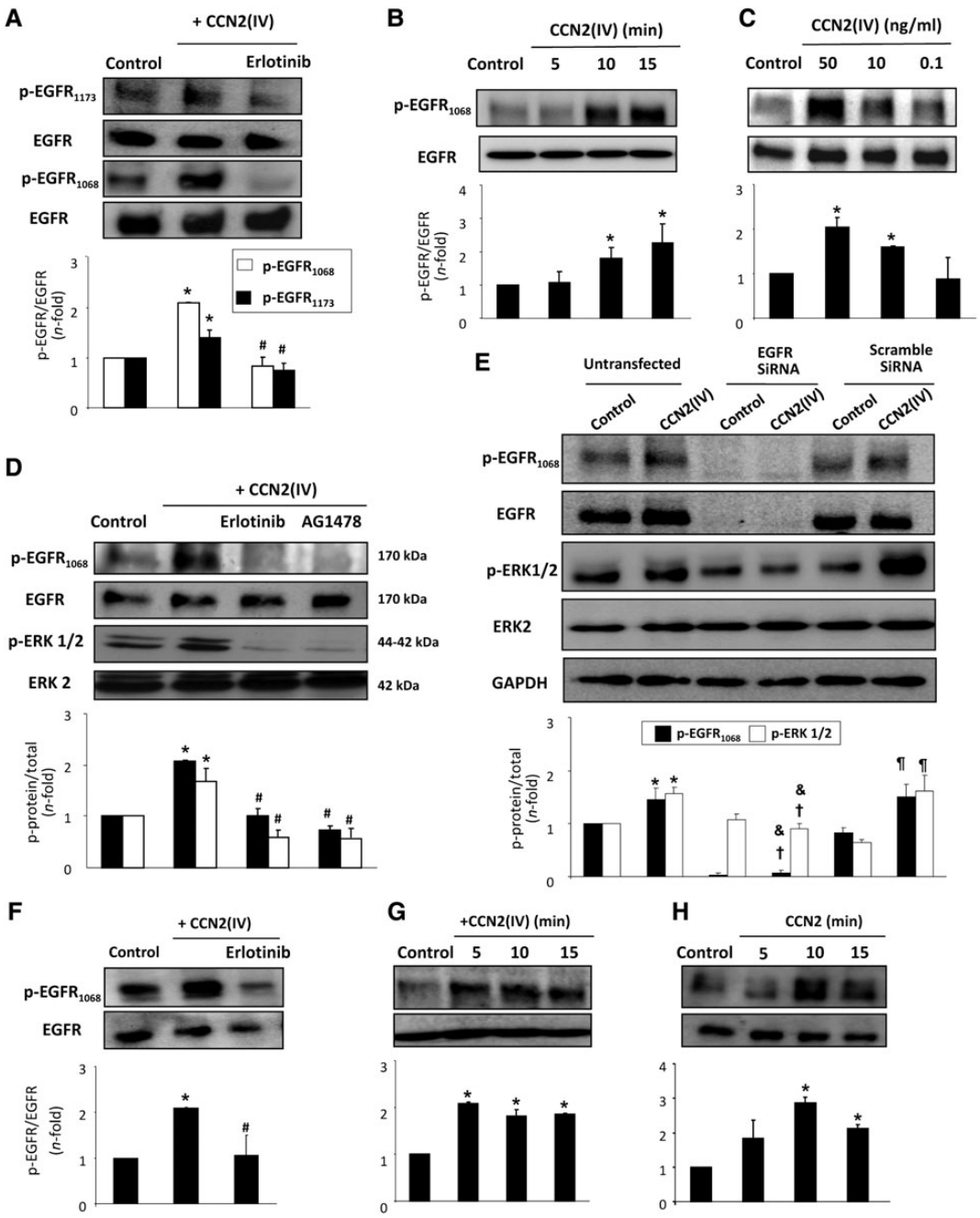

Figure 2 CCN2 activates EGFR signalling in renal cells. EGFR activation was evaluated using antibodies against phosphorylated EGFR on Y1068 (p-$EGFR_{1068}$) or Y1173 (p-$EGFR_{1173}$), both implicated in ERK activation. CCN2(IV) increased EGFR phosphorylation in human (**A**) and murine (**B**–**D**) tubular cells. (**A**) Human tubular epithelial cells (HK2 cells) were treated with 50 ng/ml CCN2(IV) for 15 min. Some cells were pre-incubated with erlotinib (10 μmol/L). Murine tubular epithelial cells were treated with 10 ng/ml CCN2(IV) for increasing time periods (**B**) or with several concentrations of CCN2(IV) (range 50–0.1 ng/ml) for 15 min (**C**). (**D**) Cells were pre-incubated for 1 h with erlotinib (10 μmol/L) or AG1478 (100 nmol/L) before the stimulation with 10 ng/ml CCN2(IV) for 15 min. (**E**) CCN2(IV) induces EGFR phosphorylation linked to ERK activation in tubular epithelial cells. HK2 cells were incubated with transfection reagent alone (untransfected) or transfected with EGFR siRNA or scramble siRNA, and then treated or not with CCN2(IV). ERK activity was determined by levels of phosphorylated ERK1/2. Total EGFR, ERK, and GAPDH levels were used as loading/silencing controls. Data of phosphorylated protein vs. total protein levels are expressed as mean ± SEM of 8 independent western blot experiments. $^{*}P < 0.05$ vs. control-untransfected. $^{\P}P < 0.05$ vs. untreated scramble siRNA-transfected cells. $^{\dagger}P < 0.05$ vs. CCN2(IV)-treated scramble siRNA-transfected cells. $^{\&}P < 0.05$ vs. CCN2(IV)-treated untransfected cells. Renal fibroblasts (**F**) and human mesangial cells (**G**) were treated with 10 ng/ml CCN2(IV) for 15 min and 50 ng/ml CCN2(IV) for increasing time periods (5, 10, and 15 min), respectively. (**H**) Full-length CCN2 activates the EGFR pathway. Murine tubular epithelial cells were stimulated with 34 ng/ml CCN2 (full-length recombinant protein) for increasing time periods. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. CCN2(IV) alone. Figures (except **E**) show a representative western blot experiment and data are expressed as mean ± SEM of 5–8 independent experiments.

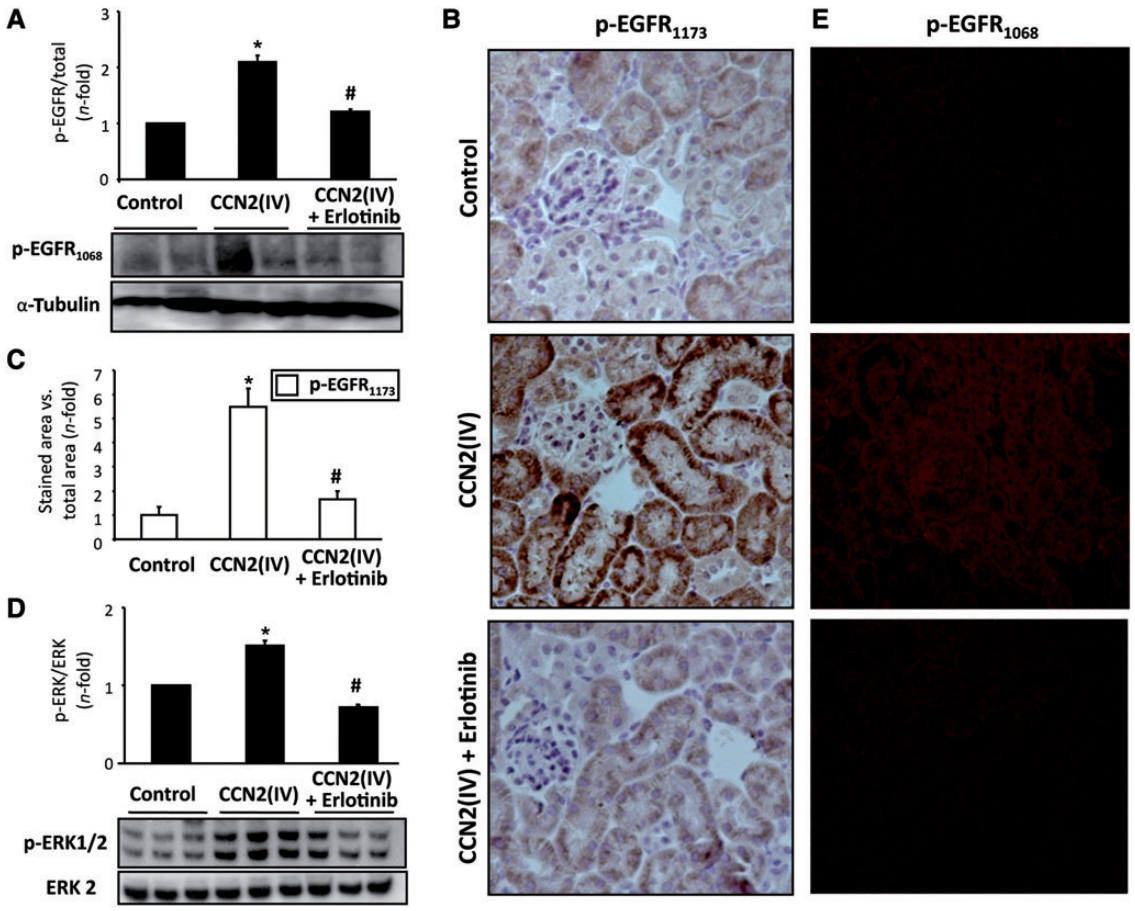

Figure 3 CCN2 induces EGFR phosphorylation in the kidney. (**A**) C57BL/6 mice were i.p. injected with 2.5 ng/g of body weight of recombinant CCN2(IV) or vehicle (saline) and sacrificed 24 h later. Some animals were treated with erlotinib (40 mg/kg per day) or its vehicle (control group), starting 24 h before CCN2(IV) injection. EGFR activation was determined in total renal extracts by western blot analysis. Figure shows two representative mice from each group and data are expressed as mean ± SEM of 8–10 mice per group. The localization of activated EGFR was determined by immunohistochemistry using antibody against p-EGFR$_{1173}$ (**B**) and by immunofluorescence using antibody against p-EGFR$_{1068}$ (**E**), which showed increased p-EGFR immunostaining mainly in tubular epithelial cells. (**C**) The pEGFR$_{1173}$ immunostaining in **B** was quantified and expressed as mean ± SEM of 8–10 animals per group. (**D**) ERK activation is a downstream mechanism of CCN2/EGFR signalling in the kidney. Figure shows a representative experiment and data of p-ERK levels are expressed as mean ± SEM of 8–10 mice per group. *$P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. CCN2(IV).

cells) in the kidney to levels similar to control mice (Figure 4A and B). EGFR inhibition also down-regulated renal gene expression and protein levels of several proinflammatory factors (CCL-2 and IL-6) to control levels (Figure 4C and D).

In cultured murine tubular epithelial cells, CCN2(IV) regulates some proinflammatory factors (Sanchez-Lopez et al., 2009). The blockade of EGFR by erlotinib or AG1478 diminished CCN2(IV)-induced gene overexpression and protein release of CCL-2 and IL-6 to control levels (Figure 4E and F). Similar inhibitory effect was found by EGFR gene silencing (Figure 4G). These data link EGFR activation by CCN2(IV) with the up-regulation of proinflammatory factors in tubular epithelial cells and the inflammatory response observed in the kidney.

ADAMs are not involved in CCN2-mediated EGFR-signalling activation

Besides direct activation of EGFR by ligand binding, several factors can indirectly activate EGFR by a process termed 'transactivation'. EGFR transactivation is regulated by ADAMs, disintegrins, and matrix metalloproteases (MMPs) that mediate EGFR ligand shedding (Ohtsu et al., 2006). In renal cells, ADAM-17 regulates EGFR transactivation (Lautrette et al., 2005; Wolf, 2005). We have observed that a pan-specific inhibitor of MMPs, GM6001, did not modify CCN2(IV)-induced EGFR phosphorylation (Figure 5A). Moreover, the pharmacological inhibition of ADAM-17 using TAPI-2 or ADAM-17 gene silencing did not modify CCN2(IV)-induced EGFR phosphorylation (Figure 5A and B). These data clearly demonstrate that ADAMs are not involved in CCN2-mediated EGFR-signalling activation, and support our findings that CCN2 directly interacts with EGFR.

Role of integrins in CCN2-induced EGFR activation

Integrins are heterodimeric receptors for cell-surface adhesion molecules and extracellular matrix proteins, which are composed of two subunits, α and β. Each αβ combination has specific signalling properties (Juliano, 2002). To date, eighteen α and eight β subunits have been identified, which form at least 24 different αβ integrins (Humphries et al., 2006). Integrin-binding sites are present in CCN2 and mediate several effects (Chen et al., 2004; Gao and Brigstock, 2005). We first tested the involvement of integrins in EGFR activation

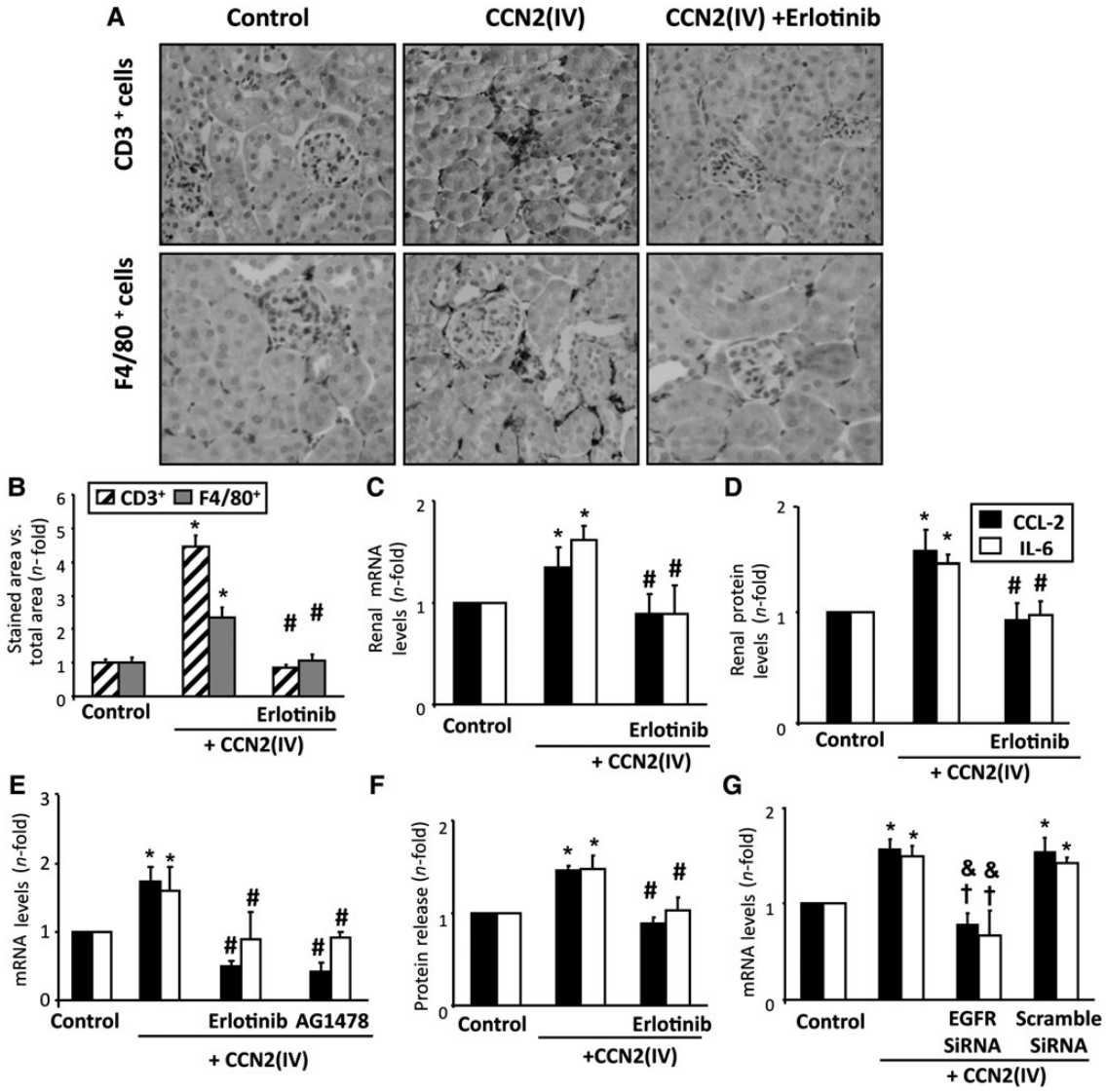

Figure 4 EGFR kinase inhibition decreases CCN2(IV)-induced renal inflammatory cell infiltration. In paraffin-embedded kidney sections, immunohistochemistry using anti-F4/80 and anti-CD3 was performed to characterize monocyte/macrophages and T lymphocytes, respectively (representative sections from each group, **A**; staining quantification, **B**). Magnification 200×. Erlotinib inhibits CCN2(IV)-induced up-regulation of renal inflammatory molecules. CCL-2 and IL-6 gene expression levels were determined by real-time PCR (**C**) and protein levels by ELISA (**D**) in total renal extracts from different animal groups. Data are expressed as mean ± SEM of 8–10 animals per group. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. CCN2(IV). (**E**–**G**) CCN2(IV) increases proinflammatory factors via EGFR activation in murine tubular epithelial cells. Cells were pre-incubated for 1 h with erlotinib (10 μmol/L) or AG1478 (100 nmol/L) before the stimulation with 10 ng/ml CCN2(IV) for 6 h (gene studies; **E**) or 24 h (protein studies, cell-conditioned medium; **F**). (**G**) EGFR gene silencing inhibits upregulation of proinflammatory molecules caused by CCN2(IV) in human tubular epithelial cells. HK2 cells were transfected or not with an EGFR or scramble siRNA before the stimulation with 50 ng/ml CCN2(IV) for 6 h (gene studies). Data are expressed as mean ± SEM of 6 (**E**), 5 (**F**), and 4 (**G**) independent experiments. $^{*}P < 0.05$ vs. control-untransfected. $^{\#}P < 0.05$ vs. CCN2(IV). $^{\dagger}P < 0.05$ vs. CCN2(IV)-treated scramble siRNA-transfected cells. $^{\&}P < 0.05$ vs. CCN2(IV)-treated untransfected cells.

by CCN2(IV) using arginine-glycine-aspartic acid (RGD) peptides. RGD was originally identified as the sequence in fibronectin that is a recognition site for α5β1 integrin, but it also recognizes α3β1, α8β1, αVβ1, αVβ3, αVβ5, αVβ6, and α2bβ3 integrins (Plow et al., 2000). Pre-incubation of HK2 cells with cyclic RGD peptide (GRGDSP), but not with control peptide (GRGDESP), reduced CCN2(IV)-induced EGFR activation (Figure 6A), suggesting that integrins with RGD recognition specificity may be involved in CCN2 responses.

The specific integrins involved in CCN2 actions are cell-dependent. In hepatic stellate cells, αVβ3 integrin interacts with CCN2(IV) (Gao and Brigstock, 2004), while in pancreatic stellate cells it is α5β1 (Gao and Brigstock, 2005). Therefore, we tested the involvement of these two integrins in tubular epithelial cells. Pre-incubation of HK2 cells with a neutralizing antibody against αVβ3 integrin inhibited CCN2(IV)-induced EGFR phosphorylation, while a control IgG or a α5β1-neutralizing antibody had no effect (Figure 6B). Using siRNA against β3 or αV integrins showed

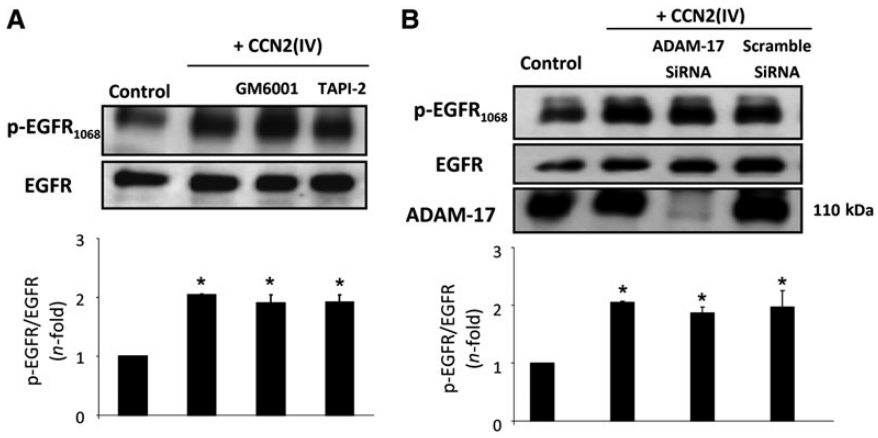

Figure 5 Pharmacological inhibition of MMPs or ADAM-17 and gene silencing of ADAM-17 do not modify CCN2(IV)-induced EGFR phosphorylation in human tubular epithelial cells. (**A**) HK2 cells were pre-incubated for 1 h with the pan-specific MMPs inhibitor GM6001 (1 μmol/L) or the ADAM-17-specific inhibitor TAPI-2 (20 μmol/L) before the stimulation with 50 ng/ml CCN2(IV) for 15 min. Values are mean ± SEM from at least 6 independent experiments. *$P < 0.05$ vs. control. (**B**) HK2 cells were transfected with ADAM-17 siRNA or scramble siRNA, and then treated with 50 ng/ml CCN2(IV) for 15 min. Values are mean ± SEM from at least 4 independent experiments. *$P < 0.05$ vs. control-untransfected.

similar inhibitory effects, while a scrambled siRNA had no effect (Figure 6C and D). These data clearly show that αVβ3 integrin mediates CCN2(IV)-induced EGFR activation.

Next, we evaluated whether CCN2(IV) could directly bind to αVβ3 integrin by IP experiments. In CCN2(IV)-treated HK2 cells, the formation of CCN2-β3 and CCN2-αV complexes was found (Figure 6E). Moreover, EGFR gene silencing did not modify the CCN2(IV) binding to αV or β3 integrin subunits (Figure 6E), demonstrating the direct binding of CCN2(IV) to αVβ3 integrin, even in the absence of EGFR. Interestingly, pre-incubation of cells with the neutralizing antibody against αVβ3 integrin did not modify the CCN2–EGFR complex formation, assessed by EGFR co-IP upon CCN2(IV) treatment (Figure 6F). These data showed that αVβ3 integrin directly binds to CCN2(IV), but is not necessary for the binding of CCN2(IV) to EGFR and the subsequent complex formation.

Potential crosstalk between EGFR and TrkA in response to CCN2(IV) stimulation in cultured tubular epithelial cells

In mesangial cells, CCN2 stimulated tyrosine phosphorylation of proteins at 75–80 and 140–180 kDa within 10 min, and previous studies have identified the neurotrophin receptor TrkA (molecular weight ~140 kDa) as a potential CCN2 receptor (Wahab et al., 2005), also in other cell types, such as cardiomyocytes (Wang et al., 2010). Therefore, the role of TrkA in CCN2-induced responses in tubular epithelial cells was evaluated. Western blot was performed using an antibody that recognizes TrkA phosphorylated on Y490, previously related to CCN2 responses in mesangial cells (Wahab et al., 2005). We found that CCN2(IV) increased TrkA phosphorylation levels in HK2 cells, which was abolished in TrkA-silenced cells (Figure 7A), showing the specificity of this CCN2(IV) response.

Next, we further evaluated the potential interrelation between EGFR and TrkA in HK2 cells. Gene silencing of TrkA diminished CCN2(IV)-induced EGFR phosphorylation (Figure 7A). Moreover, pharmacological inhibition of TrkA using K252a also blocked CCN2(IV)-mediated EGFR activation (Figure 7B). On the other hand, EGFR gene silencing inhibited TrkA phosphorylation induced by CCN2(IV) (Figure 7C). These data indicate an EGFR/TrkA receptor crosstalk.

CCN2 is a downstream mediator of TGF-β-induced EGFR activation

CCN2 is a downstream mediator of TGF-β-induced profibrotic responses (Ruiz-Ortega et al., 2007). In HK2 cells, blockade of endogenous CCN2 production by specific CCN2 gene silencing markedly diminished TGF-β-induced EGFR phosphorylation after 24 h of TGF-β incubation, compared with scramble siRNA controls (Figure 8). Our results confirm and extend previous data, showing that CCN2 is a downstream mediator of TGF-β-induced responses, including EGFR signalling.

Discussion

By surface plasmon resonance, we have detected direct binding of CCN2 to the immobilized extracellular fraction of EGFR. Interestingly, both full-length CCN2 and CCN2(IV) bound to EGFR and increased EGFR phosphorylation in cultured renal cells, suggesting that the EGFR-binding site is present in the carboxyl-terminal module. Our *in vivo* studies show that CCN2(IV) activates EGFR/ERK pathway in the kidney, mainly in tubular epithelial cells. Our *in vitro* studies in these cells demonstrate that CCN2(IV) rapidly binds to the cellular membrane and leads to CCN2–EGFR complex formation, increases EGFR phosphorylation, and activates downstream signalling mechanisms. Seven ligands for EGFR have been identified so far: EGF, TGF-α, heparin binding EGF-like growth factor, amphiregulin, betacellulin, epigen, and epiregulin (Dreux et al., 2006). Data presenting here extend this list, suggesting that CCN2 is another ligand for EGFR.

Ligand binding to EGFR induces a conformational change leading to the formation of receptor homo- or heterodimers and subsequent activation of the intrinsic tyrosine kinase domain by phosphorylation of specific tyrosine residues within the cytoplasmic tail of the receptor (Sweeney and Carraway, 2000). Phosphorylation of different tyrosine residues occurs upon binding of different ligands to the same EGFR, leading to a variety of downstream signal transduction pathways that can be selectively activated (Sweeney and Carraway, 2000). Our *in vivo* data clearly demonstrated that CCN2(IV) administration activated renal EGFR, as shown by increased EGFR phosphorylation, mainly in tubular epithelial cells. In these cells *in vitro* and *in*

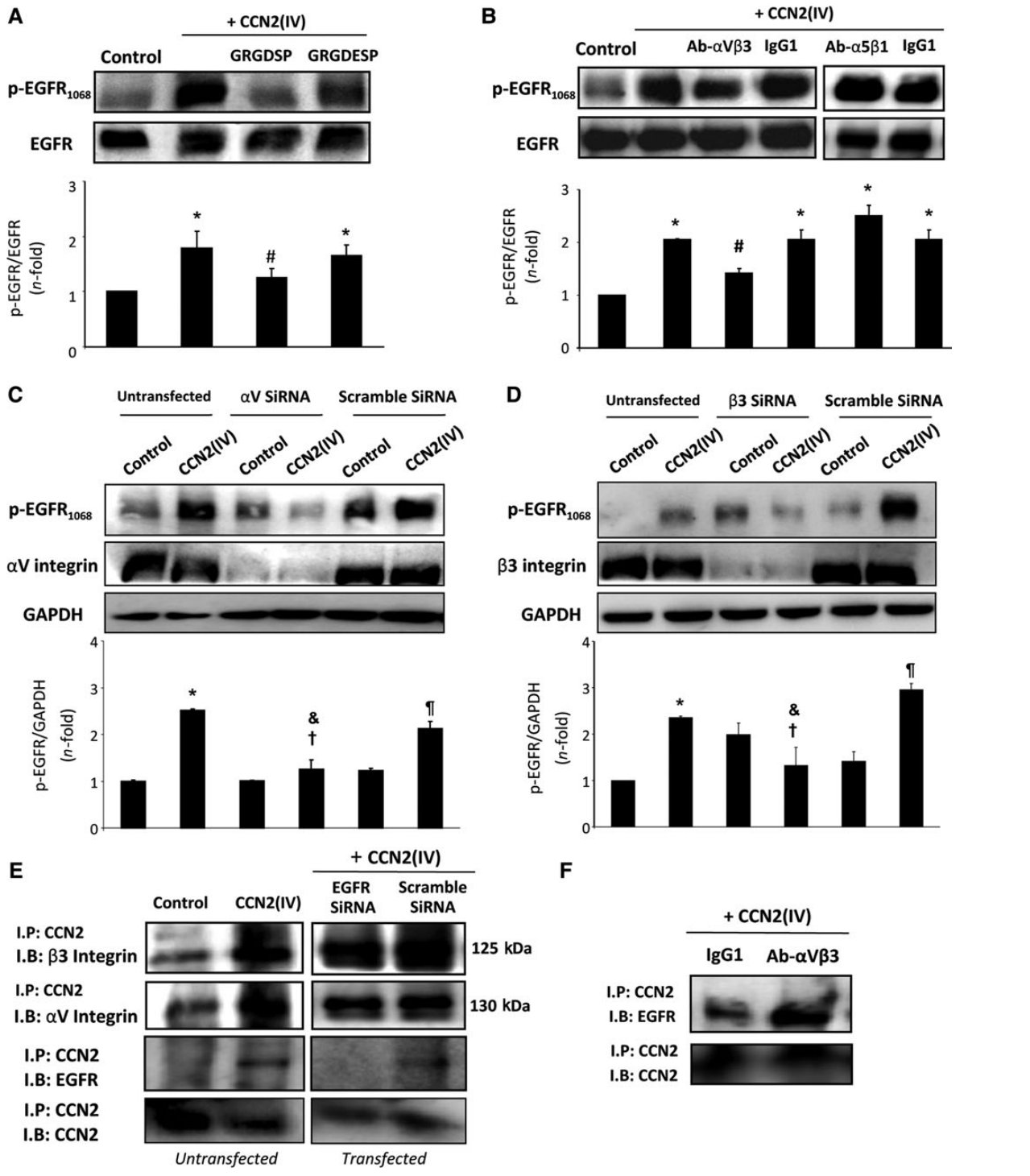

Figure 6 Role of integrins in CCN2(IV)-induced EGFR activation in cultured human tubular epithelial cells. HK2 cells were pre-incubated with 0.2 mmol/L RGDSP or its control peptide RGDESP (**A**) or with 5 μg/ml neutralizing antibodies against αVβ3 integrin, α5β1 integrin, or their corresponding IgG1 control (**B**). $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. CCN2(IV). HK2 cells were non-transfected or transfected with αV (**C**), β3 (**D**), or scramble siRNA, and then treated or not with 50 ng/ml CCN2(IV) for 15 min. Figures show a representative western blot and data are expressed as mean ± SEM of 8 independent experiments. αV or β3 integrin was used as silencing control and GAPDH as loading control. $^{*}P < 0.05$ vs. control-untransfected. $^{\P}P < 0.05$ vs. untreated scramble siRNA-transfected cells. $^{\dagger}P < 0.05$ vs. CCN2(IV)-treated scramble siRNA-transfected cells. $^{\&}P < 0.05$ vs. CCN2(IV)-treated untransfected cells. (**E**) EGFR gene-silenced cells were stimulated with CCN2(IV) and cross-linked, and cell lysates were immunoprecipitated with anti-CCN2 antibody (IP) and analysed by western blot (IB) with antibodies against EGFR, CCN2, αV, or β3 integrins. The formation of αV/β3-CCN2(IV) complexes in the presence or absence of EGFR (blocked by gene silencing) was shown. (**F**) Cells were pre-incubated with a neutralizing αVβ3 integrin antibody or IgG control before CCN2(IV) stimulation. The αVβ3 integrin neutralization did not modify CCN2(IV)-EGFR complex formation. One representative experiment out of three with similar results was shown in **E** and **F**.

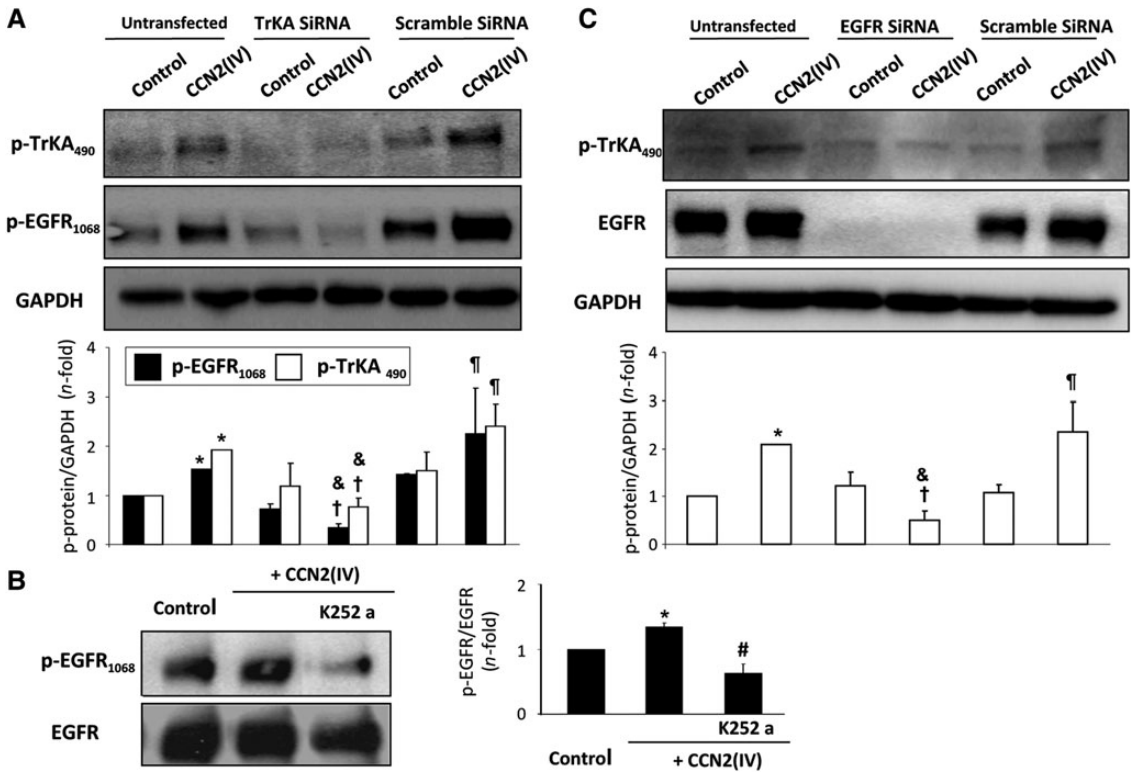

Figure 7 Blockade of TrkA by gene silencing or a specific TrkA receptor inhibitor inhibits CCN2(IV)-induced EGFR activation and EGFR gene silencing inhibits CCN2(IV)-induced TrkA activation. (**A** and **C**) HK2 cells were transfected with siRNA against TrkA (**A**) or EGFR (**C**) or its corresponding scramble. (**B**) HK2 cells were pre-incubated with 1×10^{-4} mmol/L K252a for 1 h. In all experiments, cells were treated or not with 50 ng/ml CCN2(IV) for 15 min. Activation of EGFR or TrkA was evaluated using specific p-EGFR$_{1068}$ or p-TrkA$_{490}$ antibodies. EGFR and pTrkA were used as silencing controls and GAPDH as loading control. Figures show a representative western blot experiment and data are expressed as mean ± SEM of 4 (**A**), 3 (**B**), and 4 (**C**) independent experiments. $^{*}P < 0.05$ vs. control-untransfected. $^{¶}P < 0.05$ vs. untreated scramble siRNA-transfected cells. $^{†}P < 0.05$ vs. CCN2(IV)-treated scramble siRNA-transfected cells. $^{\&}P < 0.05$ vs. CCN2(IV)-treated untransfected cells.

vivo, CCN2(IV) induced EGFR phosphorylation on Y1068 and Y1173, which have been previously associated to MAPK cascade (Rojas et al., 1996; Sweeney and Carraway, 2000). In cultured tubular epithelial cells, EGFR blockade by gene silencing or kinase inhibition inhibited CCN2(IV)-induced ERK activation and upregulation of proinflammatory genes. *In vivo* treatment with erlotinib markedly diminished the number of inflammatory cells, the up-regulation of proinflammatory markers, and ERK activation in the kidneys of CCN2(IV)-injected mice. Our results suggest that CCN2(IV) directly binds to EGFR and activates its signalling pathway leading to the modulation of downstream mechanisms, such as ERK activation, and cellular responses, including renal inflammatory cell infiltration.

EGF ligands exist as inactive transmembrane precursors, requiring ADAM-mediated proteolytic cleavage of their ectodomain to be released as mature soluble ligands, whereby ADAMs regulate EGFR ligands availability (Melenhorst et al., 2008). Regarding CCN2, the full-length protein can be digested by proteases including MMPs. In particular, MMP-2 leads to the generation of a 11 kDa carboxyl-terminal fragment, which corresponds to CCN2(IV) (Hashimoto et al., 2002; De Winter et al., 2008; Tam et al., 2009). In the urine of patients with diabetic nephropathy, full-length CCN2 and CCN2(IV) were both found (Riser et al., 2003). However, the mechanisms involved in the regulation of CCN2 degradation in renal diseases are unknown and future studies are needed.

EGFR transactivation is mediated by ADAM-dependent EGFR ligand shedding by factors that bind G protein-coupled receptors (Ohtsu et al., 2006). Depending on the tissue, different ADAMs may be involved in EGFR ligand shedding. In this sense, Angiotensin II-induced EGFR transactivation in the kidney is regulated by ADAM-17 (Lautrette et al., 2005; Wolf, 2005; Flamant et al., 2012), while ADAM-12 mediates this process in the heart (Asakura et al., 2002). We have observed that the pharmacological inhibition of MMPs or ADAM-17 and gene silencing of ADAM-17 did not modify CCN2(IV)-induced EGFR phosphorylation in renal cells, demonstrating that MMPs are not involved in CCN2(IV)-mediated EGFR-signalling activation and confirming the direct interaction of CCN2(IV) with EGFR.

Several *in vitro* studies have shown that CCN2, through its binding to different domains, regulates different processes. The aminus terminal portion binds IGF-I and synergizes in the production of matrix proteins in renal cells (Kim et al., 1997; Wang et al., 2001; Lam et al., 2003). In *Xenopus* cells, CCN2, through the cysteine-rich domain, directly binds to TGF-β and acts as a cofactor that enhances TGF-β binding to its receptors and Smad-responsive promoter activation (Abreu et al., 2002). TSP-1 domains have been implicated in the binding to extracellular matrix proteins, integrins, heparan sulphate proteoglycans, low-density lipoprotein receptor-related protein, and vascular endothelial growth factor (Adams and Tucker, 2000; Segarini et al., 2001; Inoki et al., 2002; Leask and Abraham, 2006;

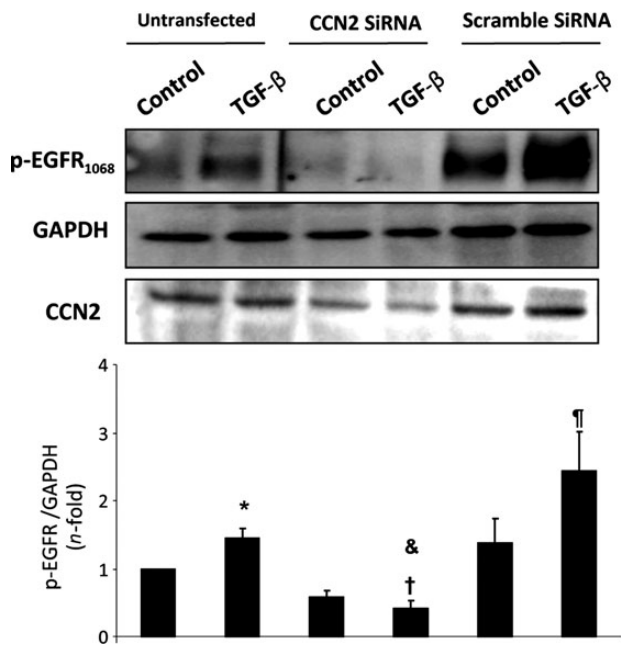

Figure 8 Blockade of endogenous CCN2 production diminishes TGF-β-induced EGFR activation. CCN2 gene silenced HK2 cells and control cells were stimulated with 1 ng/ml TGF-β for 24 h. Figure shows a representative experiment and data are expressed as mean ± SEM of 3 independent western blot. $^{*}P < 0.05$ vs. control-untransfected. $^{¶}P < 0.05$ vs. untreated scramble siRNA-transfected cells. $^{†}P < 0.05$ vs. TGF-β-treated scramble siRNA-transfected cells. $^{\&}P < 0.05$ vs. TGF-β-treated untransfected cells.

Chen and Lau, 2010). The carboxyl-terminal CCN2 cystine-knot module binds to integrins and exerts additional signalling capabilities, including regulation of fibrosis and inflammation (Leask and Abraham, 2006; Liu et al., 2006; De Winter et al., 2008). Our *in vitro* data show that this carboxyl-terminal fragment binds to EGFR leading to the regulation of proinflammatory factors. Our findings implicate integrins as key mediators of CCN2(IV)-induced EGFR activation using RGD peptides and provide evidence that integrin αVβ3 is involved in CCN2(IV)-induced EGFR activation based on results of the *in vitro* experiments utilizing neutralizing anti-integrin antibodies and siRNA. The ability of integrins to cooperate with receptor tyrosine kinases, including EGFR, to transduce proliferative signals and regulate cell survival and migration has been discussed previously (Miranti and Brugge, 2002; Schwartz and Ginsberg, 2002). Integrins are able to form physical complexes with EGFR at the cell membrane and trigger ligand-independent phosphorylation of Y845, Y1068, Y1086, and Y1173 residues in the EGFR molecule (Moro et al., 1998). This integrin-dependent EGFR activation appears necessary for full EGFR-dependent transcriptional responses (Cabodi et al., 2004). Our data show that αVβ3 integrin binds to CCN2(IV) and is involved in EGFR-signalling transduction, but is not necessary for its binding to the EGFR and the formation of EGFR-CCN2(IV) complex.

TrkA is a member of the Trk family of cell membrane receptors (TrkA, TrkB, and TrkC). These receptors interact with neurotrophins and form homodimers or heterodimers with the low-affinity pan neurotrophin receptor, p75NTR. Neurotrophins, such as nerve growth factor, brain-derived neurotrophic factor, neurotrophin 3 and 4, and their receptors, are important for the development, survival, and function of neurons (Allen and Dawbarn, 2006). The neurotrophin receptor TrkA has been proposed as a CCN2 receptor in mesangial cells (Wahab et al., 2005) and involved in diabetic nephropathy (Fragiadaki et al., 2012). In murine cardiomyocytes, CCN2 via TrkA induced profibrotic and proinflammatory effects (Wang et al., 2010). Interestingly, cardiomyocytes express additional CCN2 receptors that mediate proinflammatory actions, since CCN2-induced TNF-α and IL-6 mRNA upregulation occurred in the absence of TrkA (Wang et al., 2010). In tubular epithelial cells, we have found that CCN2(IV) activates TrkA signalling. Gene silencing and pharmacological inhibition of TrkA diminished EGFR phosphorylation, and EGFR gene silencing inhibited TrkA phosphorylation induced by CCN2(IV), demonstrating EGFR/TrkA crosstalk in response to CCN2(IV) stimulation. The similarity of phosphoproteomic profiles between TrkA and EGFR indicates a considerable overlap in downstream signallings originated in these tyrosine kinase receptors (Bradshaw et al., 2013). In monocytes, EGFR/TrkA crosstalk has been described in response to G protein-coupled receptors and linked to modulation of proinflammatory mediators (El Zein et al., 2010). The complexity of tyrosine kinase receptor signalling and interactions will require future studies.

The incidence of chronic kidney disease is increasing and current treatments only retard disease progression. Many studies using different strategies for blocking CCN2 activity have proven beneficial effects on experimental pathologies, including renal diseases (Leask and Abraham, 2006) However, these are far from being used in humans. CCN2 overexpression has been described in a wide variety of progressive human renal diseases and has been proposed as a risk biomarker (Riser et al., 2003; Tam et al., 2009; Slagman et al., 2011). We describe here that CCN2 (both full-length and the carboxyl-terminal fragment) interacts with and activates EGFR, leading to ERK activation and regulation of renal inflammation. CCN2 has been described as a downstream mediator of profibrotic factors (Hashimoto et al., 2002; Ruperez et al., 2003; Leask and Abraham, 2006). Our results showing that CCN2 gene silencing inhibited EGFR pathway activation in response to TGF-β support these findings and extend the importance of EGFR signalling in the fibrotic process. Experimental evidences suggest that EGFR inhibition may have therapeutic potential for kidney diseases (Lautrette et al., 2005; Flamant et al., 2012; Liu et al., 2012). Our findings indicate that CCN2 is a new ligand of the EGFR and identify this receptor as an important therapeutic target for renal diseases.

Materials and methods

Cell cultures

Human renal proximal tubular epithelial cells (HK2 cell line, ATCC CRL-2190) were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin transferrin selenium (ITS), and 36 ng/ml hydrocortisone in 5% CO_2 at 37°C. Murine renal cortical fibroblasts (TFB cell line) and murine proximal tubular epithelial cells [murine tubular-epithelial (MCT) cell line] originally obtained from Dr Eric Neilson (Vanderbilt University) were grown in RPMI with 10% FBS, 2 mmol glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. At 60%–70% of confluence, HK2 and TFB cells were growth-arrested in serum-free medium for 24 h

before the experiments, while MCT cells were maintained in 1% FBS.

Reagents

The full-length molecule of CCN2 (CCN2; Biovendor), TGF-β and CCN2(IV) (Preprotech), erlotinib (Vichem), tyrphostin AG1478 (Alomone Labs), K-252a and GM6001 (Calbiochem), TAPI-2 (Enzo Life Sciences), RGDs peptides (Bachem), and neutralizing. Neutralizing antibodies against integrin αVβ3, integrin α5β1, and IgG1 (Millipore) were used. DMSO, used as solvent of some reagents, had no effect on cell viability or gene expression.

Ligand–receptor interaction assays

Surface plasmon resonance interaction analysis was performed using Biacore 3000 (GE Healthcare). Data were collected using the highest collection rate. All experiments were carried out at 25°C using HBS-EP (10 mmol/L HEPES, 150 mmol/L NaCl, 3 mmol/L EDTA, 0.005% P20, pH 7.4) as running buffer. CM5 sensor chip, *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC), and ethanolamine HCl were obtained from GE Healthcare. EGFR extracellular domain (Genway) was immobilized on the surface of a CM5 sensor chip by the standard amino coupling procedure at a flow rate of 5 μl/min. The surface was activated for 7 min using a 0.05 mol/L NHS/0.2 mol/L EDC mixture. Then, 5 μg/ml EGFR in 10 mmol/L sodium acetate (pH 5.0) was injected for 13 min. Finally, residual activated groups on the surface were deactivated by a 7 min injection of 1 mol/L ethanolamine (pH 8.5). Immobilization density reached 500 RU. An additional flow cell was activated and deactivated and then used as a reference surface. For kinetic analysis, CCN2 and CCN2(IV) were diluted in HBS-EP (range 1–250 mmol/L). Concentration series and blank samples were injected for 3 min using a flow rate of 50 μl/min and the dissociation was monitored for 3 min. Data processing and kinetic analysis were performed using BiaEvaluation 4.1.1. (GE Healthcare). Data were double referenced using reference surface subtraction and blank correction. Processed data were globally fit to a simple 1:1 interaction model.

Live cell confocal microscopy

Cells were imaged using a Leica TCS SP5 confocal microscope. Fluorophore Cy-5-emitted fluorescence was monitored with a 550 ± 2 nm band pass or a 670 nm long pass filter and DAPI was excited using a DIODE laser. For video rate confocal, the images were captured (1 frame every 1.33 sec) at 400 Hz for a period of 2 min and digitalized using the LIF/LEICA program (LEICA microsystems). CCN2 fluorescence by Cy5 labelling was detected using Cy-5 fluorophore (1 nmol/μl; Amersham) following the manufacturer instructions.

Chemical cross-linking

Chemical cross-linking was carried out as previously described, using DTSSP (Pierce) (Ardura et al., 2010), before immunocytochemistry and co-IP experiments.

Fluorescence immunocytochemistry

Growth-arrested HK2 cells growing on glass coverslips were stimulated with CCN2(IV) or CCN2(IV)-Cy5. After chemical cross-linking, cells were fixed in 4% paraformaldehyde, washed, and blocked (PBS/10% BSA/4% serum, 1 h). Then, cells were incubated overnight with anti-EGFR (1:200 dilution; Santa Cruz Biotechnology) or anti-CCN2 (1:200 dilution; Sigma) in PBS with 1% BSA, followed by AlexaFluor®633-conjugated goat anti-mouse (red) or AlexaFluor®488-conjugated goat anti-rabbit (green) antibodies (1:300 dilution; Invitrogen), respectively. Nuclei were stained with 1 μg/ml DAPI as control for equal cell density. The absence of primary antibody was used as negative control. Samples were mounted in Mowiol 40–88 (Sigma) and examined by using a Leica DM-IRB confocal microscope.

Co-IP assays

After chemical cross-linking, cells were lysed in 300–500 μl Triton–NP-40 lysis buffer [50 mmol/L Tris–HCl pH 8, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulphonylfluoride, 1% NP-40/IGEPAL, and a phosphatase-inhibitor cocktail (Set II, Calbiochem)], scraped off the dish, and incubated for 1 h at 4°C with shaking. Cell lysates were pre-cleared by incubating with 10 μl of protein A-agarose bead slurries (0.5 ml agarose/2 ml PBS) for 30 min at 4°C, and then centrifuged to wash away supernatants. Pre-cleared lysates were incubated with 2.5–5 μg antibody overnight at 4°C for IP experiment. The immune complexes were captured by the addition of protein A/G PLUS-agarose (20 μl) bead slurries for 1 h at 4°C. The agarose beads were collected by centrifugation, washed and subjected to SDS–PAGE, followed by western blot as described below (Ardura et al., 2010).

Western blot

Proteins were obtained from treated cells or mouse kidneys using lysis buffer (50 mmol/L Tris–HCl, 150 mol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% Triton X-100, 0.3% IGEPAL, 10 μl/ml proteinase inhibitors cocktail, 10 μl/ml PMSF, and 10 μl/ml orthovanadate). To determine protein content the BCA method was used. Cell (25 μg/lane) and kidney (100–150 μg/lane) protein extracts were separated on 6%–12% polyacrylamide-SDS gels under reducing conditions.

Samples were then transferred onto nitrocellulose membranes (BioRad), blocked with TBS/5% defatted milk/0.05% Tween-20, and incubated overnight at 4°C with the following antibodies (dilution): p-EGFR$_{1068}$ (1:250; Calbiochem), p-EGFR$_{1173}$ (1:250; Cell Signalling), p-TrkA$_{490}$ (1:1000; Cell Signalling), ADAM-17 (1:1000; Abcam), CCN2 (1:1000; Sigma), EGFR (1:250), ERK 1/2 (1:200), integrin αV (1:200), integrin β3 (1:200), pERK1/2 (1:200), and anti-phosphotyrosine PY20 (1:250; Santa Cruz). Membranes were subsequently incubated with peroxidase-conjugated IgG secondary antibody and developed using an ECL chemiluminiscence kit (Amersham). Loading controls were done using an anti-GAPDH antibody (1:10000; Chemicon) or total protein levels in phosphorylation studies. Autoradiographs were scanned using the Gel Doc™ EZ imager and analysed with the Image Lab 3.0 software (BioRad).

Gene silencing

Gene silencing in cultured cells was performed using either a pre-designed siRNA corresponding to EGFR, ADAM-17, TrkA, CCN2 (Ambion), integrin αV, or integrin β3 (Santa Cruz Biotechnology) or their corresponding scramble siRNAs. Subconfluent cells were transfected for 24 h with 25 nmol/L siRNA using 50 nmol/L Lipofectamine RNAiMAX (Invitrogen) or treated only with lipofectamine—vehicle, according to the manufacturer's instructions. Then, cells were incubated with 10% heat-inactivated FBS for 24 h, followed by 24 h in serum-free medium and then treated or not with CCN2(IV).

Animal model

Studies were performed in adult male C57BL/6 mice (9–12 weeks old, 20 g; Harlan Interfauna Ibérica) and maintained at local animal facilities. All the procedures on animals were

performed according to the European Community and Instituto de Investigación Sanitaria Fundación Jiménez Díaz Animal Research Ethical Committee guidelines. C57BL/6 mice received a single intraperitoneal injection of recombinant CCN2(IV) (endotoxin levels <0.01 units; Preprotech) dissolved in saline at the dose of 2.5 ng/g of body weight as described (Sanchez-Lopez et al., 2009), and studied 24 h later ($n = 8–10$ mice per group). To block EGFR activation, animals were treated with erlotinib (40 mg/kg/day) or its vehicle (10% Ethanol) at 24 h before CCN2 injection ($n = 8–10$ mice per group). Mice were sacrificed under anaesthesia (Ketamine-HCl/Xylazine-HCl) and then kidneys were perfused *in situ* with cold saline before removal. We have previously demonstrated that CCN2(IV) administration did not cause tubular damage or fibrosis (Sanchez-Lopez et al., 2009).

Renal histology and immunohistochemistry

Immunohistochemistry was carried out on 3 μm paraffin-embedded kidney sections. Sections were deparafinized and exposed to the PT Link (Dako) with Sodium Citrate Buffer (10 mmol/L, pH 6 or 9 depending on the immunohistochemical marker) for antigen retrieval. After endogenous peroxidase was blocked, sections were incubated with 4% BSA/8% serum in 1× wash buffer '*en vision*' (Dako) to eliminate nonspecific protein binding, followed by primary antibodies (dilution) F4/80 (1:50), CD3 (1:300; Serotec), and p-EGFR$_{1173}$ (1:200; Cell Signalling) overnight at 4°C. After washing, they were incubated with anti-IgG secondary biotinylated-conjugated antibodies (Amersham) followed by the avidin-biotin-peroxidase complex (Dako) and 3,3′-diaminobenzidine as chromogen. Sections were counterstained with Carazzi's haematoxylin.

Immunofluorescence was performed by incubating sections with 4% BSA/8% serum in PBS (for blockade), then anti-p-EGFR antibody (1:200; Dako), followed by AlexaFluor®633-conjugated antibody (1:200). The total number of positive stained cells was quantified in five randomly chosen fields (20×) using the Image-Pro Plus software. Data are expressed as positive stained area vs. total analysed area. Triplicate samples from each animal were examined in a blind manner.

ELISA for proinflammatory factors

CCL-2 and IL-6 protein levels were assayed by an ELISA kit (eBioscience), and quantified by comparison with a standard curve. Data are expressed as *n*-fold increase over the mean of control levels.

Gene expression studies

Total RNA was isolated from cells and mouse kidney samples with Trizol (Invitrogen). The cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems) using 2 μg of total RNA primed with random hexamer primers. Multiplex real-time PCR was performed using Applied Biosystems expression assays mouse CCL-2 Mm00441242_m1 and mouse IL-6 Mm00446 190_m1. Data were normalized to 18S eukaryotic ribosomal RNA 4210893E (VIC). The mRNA copy numbers were calculated for each sample by the instrument software using Ct value. Results are expressed in copy numbers, calculated relative to unstimulated cells or control mice, after normalization against 18S.

Statistical analysis

All results are expressed as mean ± SEM. Differences between agonist-treated groups and controls were assessed by Mann–Whitney test. $P < 0.05$ was considered significant. Statistical analysis was conducted using the SPSS statistical software (version 11.0).

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2. CCN2 induce TEM a través de la vía de señalización del EGFR y de NF-κB

El factor de crecimiento del tejido conectivo (CCN2/CTGF) participa en la fibrogénesis renal (Leask et al., 2009, Brigstock 2009). Estudios previos han descrito la importancia de la vía de señalización del EGFR en la patología renal (Melenhorst et al., 2008). La activación del EGFR puede desencadenar diferentes procesos celulares incluida la transición epitelio mesenquimal (TEM) en células tubuloepiteliales (Ardura et al., 2010). Este proceso de TEM contribuye a la fibrosis renal al inducir la conversión de las células túbuloepiteliales a miofibroblastos productores de matriz extracelular. Estudios previos han demostrado que en células túbuloepiteliales en cultivo, CCN2 y su fragmento de degradación C-terminal denominado (CCN2 (IV)), inducen TEM (Liu et al., 2006). En este estudio se aborda el objetivo 1 de la tesis que ha consistido en investigar los receptores y vías de señalización intracelulares implicadas en la TEM inducida por CCN2, con especial atención al EGFR y la vía de señalización del factor NF-κB. En células túbuloepiteliales de ratón se observó que el bloqueo de EGFR (mediante un inhibidor de la quinasa del receptor) revirtió la TEM inducida por CCN2 (IV), caracterizada por disminución de marcadores epiteliales, como E-cadherina y ZO-1, y a su vez una inducción de marcadores mesenquimales, como α-actina de músculo liso. Recientemente hemos descrito un *crosstalk* entre EGFR y TrKA en respuesta a la estimulación con CCN2 (Rayego-Mateos et al., 2013a). En este sentido, se observó como el bloqueo farmacológico de TrKA (K252a) inhibió la TEM inducida por CCN2 (IV) modulando marcadores epiteliales como E-cadherina. La activación de la quinasa ERK es un proceso clave en la inducción de TEM (Grotegut et al., 2006; Santibanez, 2006; Bhowmick et al., 2001) y previamente hemos descrito que se activa por CCN2/EGFR (Rayego-Mateos et al. 2013a). El tratamiento con un inhibidor de ERK (U0126) inhibió la TEM causada por CCN2 (IV), mostrando que la vía EGFR/ERK es un elemento fundamental en la TEM. NF-κB es un factor de transcripción que está implicado en la TEM asociado a procesos tumorales. El tratamiento de las células con un inhibidor de la activación de la vía canónica de NF-κB, parthenolide, o de la vía no canónica, lactacistina, revirtió la TEM inducida por CCN2(IV). El bloqueo del EGFR inhibió la activación de ambas rutas (canónica y no canónica de NF-κB) activadas por CCN2, mientras que el bloqueo de TrKA no reguló específicamente la activación de la vía canónica de NF-κB.

Estos resultados sugieren el papel clave de la activación del EGFR en proceso de TEM inducido por CCN2, y como el bloqueo de vías de señalización intracelulares como ERK y NF-κB están asociados a la modulación de la TEM inducida por este factor.

Title page

Connective tissue growth factor induces epithelial-mesenchymal transition via epidermal growth factor receptor and NF-$_K$B pathway.

Autores:

Sandra Rayego-Mateos[1], Jose Luis Morgado-Pascual[1], Adrian M. Ramos[2], Alberto Ortiz [2], Jesus Egido[3], Marta Ruiz-Ortega[1].

[1] Cellular Biology in Renal Diseases Laboratory. Universidad Autónoma Madrid. Spain.

[2] Dialysis Unit. IIS-Fundación Jiménez Díaz-UAM. School of Medicine, UAM,Madrid Spain.

[3] Renal Research Laboratory. Fundación Jiménez Díaz. Universidad Autónoma Madrid. Spain.

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Correspondence:
Marta Ruiz-Ortega
Cellular Biology in Renal Diseases Laboratory
Fundación Jiménez Díaz
Avda. Reyes Católicos, 2
28040 Madrid, Spain
Tel. 34-91-5504821
Fax. 34-91-5442636
Email:mruizo@fjd.es

ABSTRACT

Chronic kidney diseases are characterized by accumulation of extracellular matrix in the tubulointerstitial area. Fibroblasts are the main matrix-producing cells. One source of activated fibroblasts is the epithelial mesenchymal transition (EMT). Connective tissue growth factor (CCN2/CTGF) is a downstream mediator of profibrotic mediators, including TGF-β, and can induce phenotypic changes related to EMT in cultured tubular epithelial cells. We have recently described that CCN2 and its C-terminal degradation product CCN2(IV) bind to epidermal growth factor receptor (EGFR) to modulate renal inflammation. Moreover, a cross-talk between EGFR and tropomyosin receptor kinase A (TrkA) has been found. However, the receptor involved in CCN2 profibrotic actions has not been described. Our aim was to investigate whether CCN2(IV)-mediated EMT is regulated by EGFR or TrkA receptors and the downstream mechanisms involved. In cultured tubulo-epithelial cells, the blockade of EGFR, TrkA or ERK pathways, using specific pharmacological inhibitors, inhibited CCN2(IV)-induced EMT changes. CCN2(IV) activates the nuclear factor-kappa B (NF-κB) in the kidney associated to inflammation. NF-κB has been linked to EMT in cancer, but data in renal cells is scarce. In cultured tubulo-epithelial cells, CCN2(IV) activates both the canonical and the non-canonical NF-κB pathway. The blockade of both pathways, by parthenolide or lastacystin, respectively, inhibited CCN2(IV)-induced EMT changes. Interestingly, only EGFR, but not TrkA blockade, inhibited CCN2(IV)-mediated NF-κB pathway activation. Our results suggest that activation of ERK and NF-κB signaling sistems are key mechanisms involved in EMT regulation by CCN2(IV) and shows that the EGFR pathway plays a central role in CCN2 signalling.

INTRODUCTION

Regardless of the underlying etiology, most forms of chronic kidney diseases (CDK) are characterized by excessive deposition of extracellular matrix proteins (ECM) and progressive fibrosis as a final common pathway that leads to end-stage renal disease[1]. Despite intensive research, our current understanding of the precise mechanism of fibrosis is limited. Recent studies the bulk of research in this area is focused on the later stages of the fibrosis pathway, such as TGF-β, the major fibrogenic cytokine, and myofibroblasts transformation via epithelial to mesenchymal transition (EMT) or endothelial to mesenchymal transition [2-4].

Connective tissue growth factor (CCN2/CTGF) is a member of the CCN (Cyr61/CCN2/Nov) family of matricellular proteins. CCN2 is a 38-kDa cystein rich secreted protein which is upregulated in proliferative disorders or fibrotic lesions in several human diseases, including skin disorders, atherosclerosis, pulmonary fibrosis and kidney diseases [5-10]. CCN2 has been identified as a downstream mediator of the pro-fibrogenic effects of TGF-β and other factors involved in renal damage, including Angiotensin II (AngII)[11-14], and it has been suggested as a potential anti-fibrotic target [15-16]. In human biopsies of different renal pathologies and in experimental models of kidney injury, renal CCN2 overexpression was correlated with cellular proliferation and ECM accumulation, both at glomerular and interstitial areas [6,12,17]. In cultured cells recombinant CCN2 and its C-terminal degradation fragment of 10 kDa, also named CCN2(IV), significantly increase ECM production in cultured cells, including mesangial cells [18-21]. Moreover, CCN2 is one of the most important inducers of EMT [22-24] acting also as a downstream mediator of the effects of TGF-β and AngII[25]. Regarding kidney diseases, inhibition of endogenous CCN2, by antisense oligonucleotides or gene silencing, is beneficial for experimental renal diseases, including unilateral ureteral obstruction model, diabetic nephropathy and nephrectomized TGF-β1 transgenic mice [26-29] . These data suggest that CCN2 could be an important target for the treatment of renal fibrosis.

CCN2, or its proteolytic fragments, can activate multiple signalling systems, including the mitogen-activated protein kinase (MAPK) cascade and the nuclear factor-kappa B (NF-κB) pathway in several cell types, leading to regulation of cell growth, hypertrophy, inflammation and fibrosis [30-33]. CCN2 can bind to integrins and heparan sulphate proteoglycans on the cell surface and influence many different biological events [34]. Moreover, CCN2 interacts with several membrane receptors, including tyrosine receptor kinase A (TrkA) and the low density lipoprotein receptor-related protein (LRP), thus triggering intracellular signalling and downstream actions [34]. CCN2 can also interact with and modify the function of growth factors, such as TGF-β and VEGF, [35] . We have recently described that epidermal growth factor receptor (EGFR) is a functional CCN2 receptor in the kidney [31] and vascular cells[36] linked to the regulation of proinflammatory related events. In cultured tubular epithelial cells, we have described that EGFR **and** TrkA activities are mutually influenced in response to CCN2 stimulation [31]. However, the functional consequences of this crosstalk and downstream signalling pathways were not investigated.

Studies done in cancer research pointed out that modulation of NF-κB could be a potential therapeutic target by its action of EMT [37,38]. Activation of canonical NF-κB regulates snail, a transcription factor that repress E-cadherin expression, leading the dissembling of epithelial membrane and inducing EMT [39]. We have recently described that CCN2(IV) activates the canonical NF-κB signaling pathway in the kidney [21]. In the present work, our aim was to investigate the molecular mechanisms involved in CCN2(IV)-induced EMT, evaluating the role of EGFR, the potential TrKA cross-talk and downstream signaling, with special interest to the NF-κB pathway activation.

RESULTS

CCN2(IV) via EGFR induced EMT in cultured tubular epithelial cells.

Stimulation of murine tubular epithelial cells (MCTs) with 10 ng/mL CCN2(IV) for 48 hours caused a conversion of MCT cells from an epithelial to a myofibroblast-like phenotype. The transformed cells lost the typical cobblestone pattern of an epithelial monolayer, and displayed a spindle-shape, fibroblast-like morphology, assessed by phase contrast microscopy (Figure 1A). In unstimulated tubuloepithelial cells, no staining for the mesenchymal marker α-smooth muscle actin (α-SMA) was observed by confocal microscopy. Treatment with CCN2(IV) for 2 days induced α-SMA-positive microfilaments in the cytoplasm, decreased the epithelial marker zonula occludens-1 (ZO-1) and induced the nuclear localization of snail (Figure 1B). EMT changes were confirmed by western blot: CCN2(IV) diminished the epithelial marker E-cadherin, essential for the structural integrity of renal epithelium (Figure 1C). To evaluate the role of EGFR pathway cells were preincubated with two specific EGFR kinase inhibitors; erlotinib or AG1478. Both EGFR inhibitors blocked CCN2(IV)-induced changes on phenotype (Figure 1A) and on EMT–related proteins, as assessed by immunofluorescence and Western blot (Figure 1B and C).

CCN2(IV) via TRKA caused EMT in cultured tubular epithelial cells.

An EGFR and TrKA cross-talk has been previously described in CCN2 stimulated tubular epithelial cells[31] and monocytes[40]. Several studies have shown that K252a, an alkaloid-like kinase inhibitor that is known to selectively inhibit TrkA kinase activity [41], blocked CCN2(IV)-induced activation of several signaling systems, including MAPKs, and ECM production [41,42]. We found that in cultured murine tubular epithelial cells pretreatment with the TrKA inhibitor K252a restored the typical cobblestone pattern of an epithelial monolayer (Figure 2A), and prevented the E-cadherin lost caused by CCN2(IV) (Figure 2B). Moreover, CCN2(IV)-induced TrkA activation is linked to Erk activation. Prehincubation with K252a, diminished ERK 1/2 activation (Figure 2C).

ERK activation is key signaling mechanism in CCN2(IV)-induced EMT

In MCTs we have previously showed that ERK activation is a downstream mechanism of EGFR pathway activated by CCN2 (IV)[31]. CCN2 (IV) activates MAPKs cascade in several cell types [42-45]. We found that the MEK/ERK activation inhibitor, U0126, prevented CCN2(IV)-induced conversion of the epithelial to a myofibroblastic phenotype in MCT cells, as shown by phase contrast microscopy (Figure 3A), and changes in the EMT markers α-SMA and ZO-1, evaluated by confocal microscopy (Figure 3B) and the epithelial marker E-cadherin by Western blot (Figure 3C).

CCN2(IV) via EGFR, but not TrkA, activated the classical NF-κB pathway.

We have previously described that CCN2(IV) activates the classical/canonical NF-κB pathway in the kidney an in cultured MCTs[30], as we have confirmed here in human tubular epithelial cells, by increased levels of phosphorylated p65 NF-κB (Figure 4). EGFR pharmacological inhibition, by erlotinib, reduced p65 phosphorylation levels in CCN2(IV)-treated cells (Figure 4A). To further demonstrate the role of EGFR, a gene silencing approach was done. In cells transfected with EGFR siRNA, inhibition of EGFR levels were observed, and in these cells CCN2(IV) failed to increase p65 phosphorylation (Figure 4B). In contrast, TrKA blockade, by the TrKA inhibitor (K252a) or gene silencing, did not modify CCN2(IV)-induced p65 phosphorylation (Figure 5A and B). These data clearly demonstrate that EGFR but not TrkA pathway is involved in the activation of classical/canonical NF-κB pathway in cultured renal cells.

CCN2(IV) through EGFR activates the non canonical NF-κB pathway

The activation of the non-canonical NF-κB2 pathway is characterized by IKK-α phosphorylation and the modulation of p100/p52 NF-κB levels[46,47]. In human cultured tubulo-epithelial cells CCN2(IV) increased IKK-α phosphorylation and RelB nuclear levels (Figure 6A and B). EGFR blockade by erlotinib diminished CCN2(IV)-induced non-canonical NF-κB activation (Figure 6 A and B). These data clearly show that CCN2(IV) via EGFR pathway activates the non-canonical NF-κB signaling.

CCN2(IV), via NF-κB activation, induces EMT in cultured tubular epithelial cells.

To evaluate the role of NF-κB signaling in EMT, MCTs cells were preincubated with the specific inhibitors of canonical or non canonical NF-κB pathway, parthenolide or Lactacystin, respectively. Both compounds inhibited CCN2(IV)-induced changes on EMT–related proteins, as assessed by changes in the epithelial marker E-cadherin by Western Blot (Figure 7A and B).

DISCUSSION

Tubulointerstitial fibrosis is a final common pathway to end-stage chronic kidney diseases and its severity correlates with renal prognosis, therefore the investigation of novel therapeutic approaches to find anti-fibrotic targets is a very active field of research. Experimental studies have shown that EGFR blockade, using EGFR kinase inhibitors of genetic-modified mice, diminished experimental renal fibrosis[48-51]. CCN2(IV) has been documented to play a vital role in a variety of human diseases and animal models that are characterized by tissue fibrosis, including the kidney, skin, blood vessels, lung and liver[52]. In the kidney, the interaction of CCN2(IV) and tubular epithelial cell has been proven to mediate tubular cell hypertrophy and EMT[22-24].In tubular epithelial cells we have previously demonstrated that CCN2 is a downstream mediator of AngII-induced EMT, using CCN2 antisense deoxy oligonucleotides[25]. The present findings indicate that EGFR activation by CCN2(IV) can promote EMT in cultured tubuloepithelial cells. EMT is an important process that contributes to renal fibrogenesis. EMT may also contribute to the repair process of the damaged tubules. Thus, after renal injury, remnant tubuloepithelial cells de-diferentiate to mesenchymal cells which migrate towards the damaged areas, where they proliferate and subsequently differentiate into the original epithelial phenotype to restore tubular integrity [1,53]. Our *in vitro* data showing that EGFR kinase inhibition diminished EMT caused by CCN2(IV) support the idea of EGFR as an important therapeutic target to combat renal fibrosis.

CCN2 overexpression has been described in human fibrotic diseases, that it is produced temporally and spatially in close proximity to fibrotic areas [6,54,55] . Antagonists of CCN2 have proven effective in blocking pro-fibrogenic CTGF signaling pathways *in vitro* and have yielded promising data with respect to preventing experimental fibrosis[15,16]. Regarding renal diseases, knockdown of CCN2 gene expression with antisense gene transfer into rat kidney ameliorates tubulointerstitial fibrosis in obstructive nephropathy[26]. Small interfering RNA against CCN2 attenuated vascular remodeling and adventitial changes in a rat model of carotid angioplasty [56] and pulmonary vascular remodeling in cigarette smoke-exposed rats[57]. Although initial studies in fibroblasts, and other cultured cells, showed that recombinant CTGF induces ECM production [18,20,58], several *in vivo* studies have shown that CCN2 alone is not sufficient to cause ongoing fibrotic changes[59,60] . We have previously described that CCN2 did not increase TGF-β synthesis or collagen deposition in mice kidney, suggesting that CCN2 alone is not enough to induce fibrosis *in vivo*[61]. Consistent with these data, CCN2 overexpression in rat lungs only caused a temporal induction of procollagen gene expression and transient matrix accumulation, but is not sufficient to induce progressive fibrosis[60]. Podocyte-specific CCN2-transgenic mice (in C57BL/6 background) exhibited no glomerular abnormalities or proteinuria, although they are more susceptible to streptozotocin-induced diabetes[62]. However, in a model of a skin fibrosis, CCN2 alone did not induce fibrosis and only the simultaneous application of TGF-β1 caused persistent fibrosis[63]. Several studies have independently concluded that CCN2 levels (plasma or urine) could be a useful risk biomarker for renal damage in human diabetic nephropathy and other forms of CKD [64,65,66], and for cardiac dysfunction in patients exhibiting myocardial fibrosis and chronic heart failure [67]. Although these studies suggest that CCN2

could be an important target for the treatment of renal fibrosis, the use of gene silencing or antisense oligonucleotides are far from being used in humans. Our *in vitro* studies showing that the EGFR kinase inhibitor erlotinib diminished CCN2(IV)-induced EMT suggest that blockade of EGFR pathway could be a good therapeutic option to block CNN2-mediated profibrotic effects.

Previous studies have described the possibility that CCN2 can binds to several receptors. The neurotrophin receptor TrkA has been proposed such as CCN2 receptor in mesangial cells[42], and has been implicated in diabetic nephropathy[68]. CCN2 also activated TrkA in murine cardiomiocites where TrkA inhibition with a pharmacological inhibitor K252a blockaded profibrotic and proinflammatory effects induced by CCN2[69]. Some data suggest that Trks could participate in EMT. Studies done in primary cell cultures derived from malignant pleural effusions of patiens with lung adenocarcinoma, demonstrated TrkB sobreexpression in malignant cells. Moreover, TrkB receptor blockade, by using K252a or gene silencing, caused a loss of the mesenchymal marker vimentin in these cells[70]. Accordingly, we have found that pharmacological TrkA blockade (by K252a) reversed the EMT-induced changes caused by CCN2(IV) in tubular epithelial cells.

CCN2 (IV) activates MAPK cascade in several cell types[42-45] including murine tubuloepithelial cells[21], regulating many cellular responses. In murine tubuloepithelial cells, we observed that CCN2-induced MCP-1 overexpression is regulated by all three MAPKs[30]. In mesangial cells, ERK1/2 mediated CCN2-induced fibrosis, migration, cytoskeleton reorganization and chemokines production[71-73]. CCN2 promotes the proliferation and differentiation of chondrocytes, mediated by ERK and p38 MAPK[45] . In fibroblasts, ERK and JNK regulate CCN2-induced expression of α-SMA and collagen I [74], and ERK is involved in myofibroblasts proliferation[75]. In human tubuloepithelial cells, CCN2 (IV) induced the expression of integrin-linked kinase via ERK1/2 activation[76]. The activation of MAPK kinases (ERK, p38 and JNK) are very important in EMT genesis but are dependent on the cell type [77-79]. In human renal tubuloepithelial cells, ERK inhibition reversed AngII-mediated EMT[25], as observed in mesothelial cells treated by the combination of TGFβ and IL1β80. In addition, we have also showed that ERK is activated as a downstream mechanism of CCN2/EGFR signaling[31]. Now, we have found that the treatment with an inhibitor of ERK1/2 (U0126) inhibited EMT caused by CCN2 (IV). Regarding the EGFR/Trka crosstalk, data presented here show that ERK activation is a common signaling system in both EGFR and TrKa pathways. Our data confirm previous observations that remark the importance of ERK pathway as a key signaling system in EMT.

Previous studies from our group have shown that CCN2 is able to activate the classical NF-κB pathway linked to the regulation of proinflammatory events in the kidney [30]. In this work we have observed that the blockade of EGFR with a pharmacological inhibitor (Erlotinib) or gene silencing, inhibited the canonical NF-κB activation induced by CCN2(IV) in cultured renal tubuloepithelial cells. However, TrkA blockade, using the same approaches, was not able to inhibit the activation of canonical NF-κB, showing a different downstream signaling system between EGFR and TrKA.

An important finding of our paper has been to demonstrate that CCN2 activates the non-canonical NF-κB pathway. There are few stimuli known to activate the non-canonical NF-κB pathway, one of them

is TWEAK[47] .However other molecules as TNF-α do not have the capacity of activate this pathway[47]. There is little information about the role of non-canonical NF-κB2 pathway activation in kidney diseases. Studies in experimental models of renal damage, including ischemia-reperfusion and diabetic nephropathy showed elevated activation[81] and protein levels of NIK[82]. In models of acute renal toxicity damage, increased expression of RelB and p52, and upregulation of target genes (CCL-19, CCL-21) has been described[47]. Some uremic toxins like, p-cresyl sulfate and indoxyl sulfate, induce the genic expression of p52, principal NF-κB2 subunit (50-80%), in proximal tubuloepithelial cells in culture[83]. In this study we have observed as CCN2(IV) activated the non-canonical NF-κB2 pathway, that was inhibited by pharmacological inhibition of EGFR.

In summary, our in vitro studies shows that CCN2(IV) regulates EMT process by its binding and activation of the EGFR pathway, linked to activation of TrkA, ERK and NF-κB signaling systems. These data remarks the importance of EGFR pathway in CCN2 signalling.

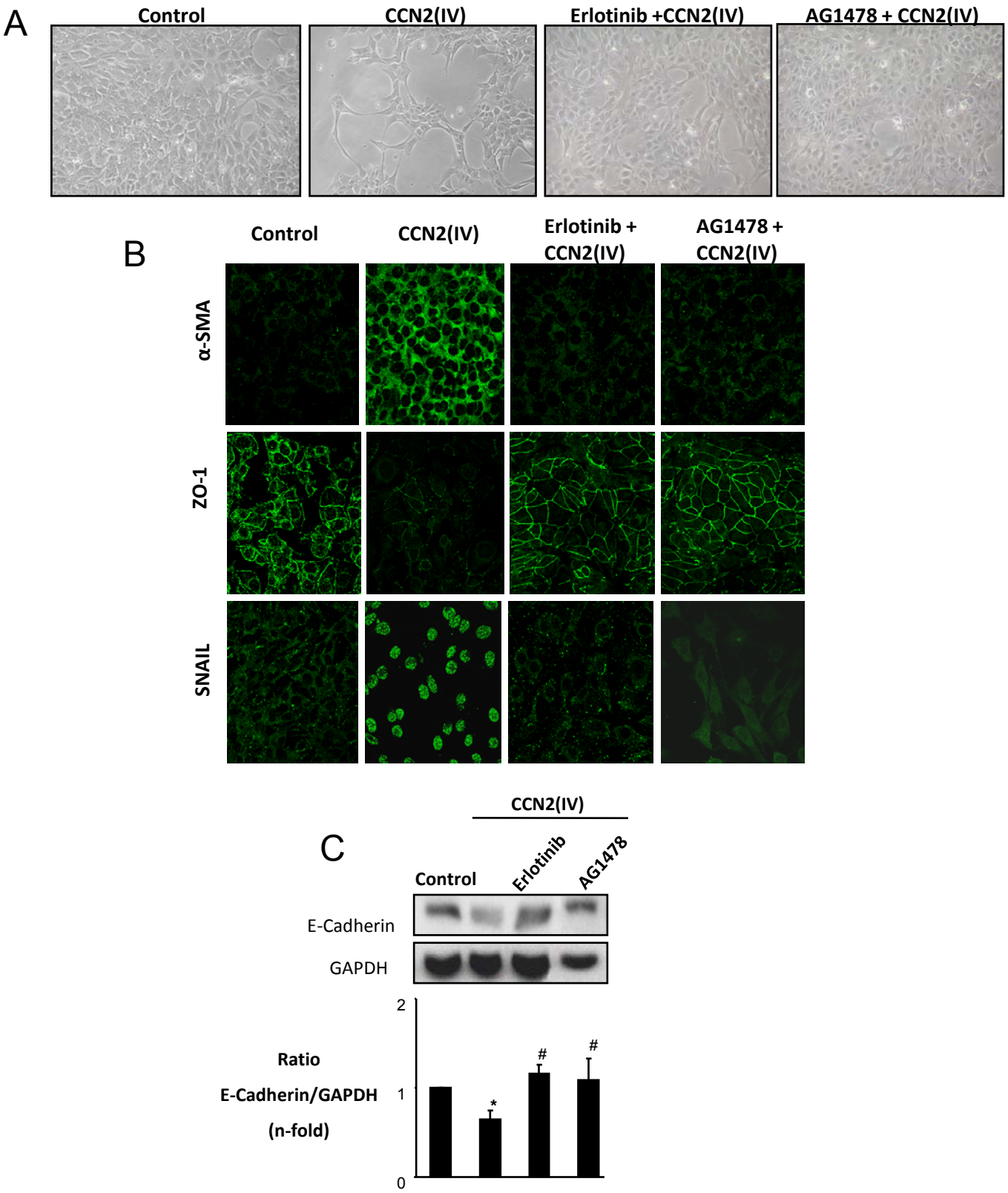

Figure 1. CCN2(IV) via EGFR can promote EMT in MCT cells. Cells were pre-incubated for 1 hour with different inhibitors: erlotinib (10 µmol/L) or AG 1478 (100 nmol/L). Then, cells were stimulated with CCN2(IV) (10 ng/mL) for 48 hours. **(A)** Phase contrast microscopy analysis of the the cells morphology **(B)** Confocal microscopy analysis of α-SMA ,ZO-1 and SNAIL immunofluorescence, using specific primary antibodies and a FITC-labeled secondary IgG was done. This represents the results of 3 independent observations. **(C)** Changes in E-cadherin protein expression were analyzed by Western blot. Representative autoradiograms are shown. Results are data ± SEM from at least 4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone.

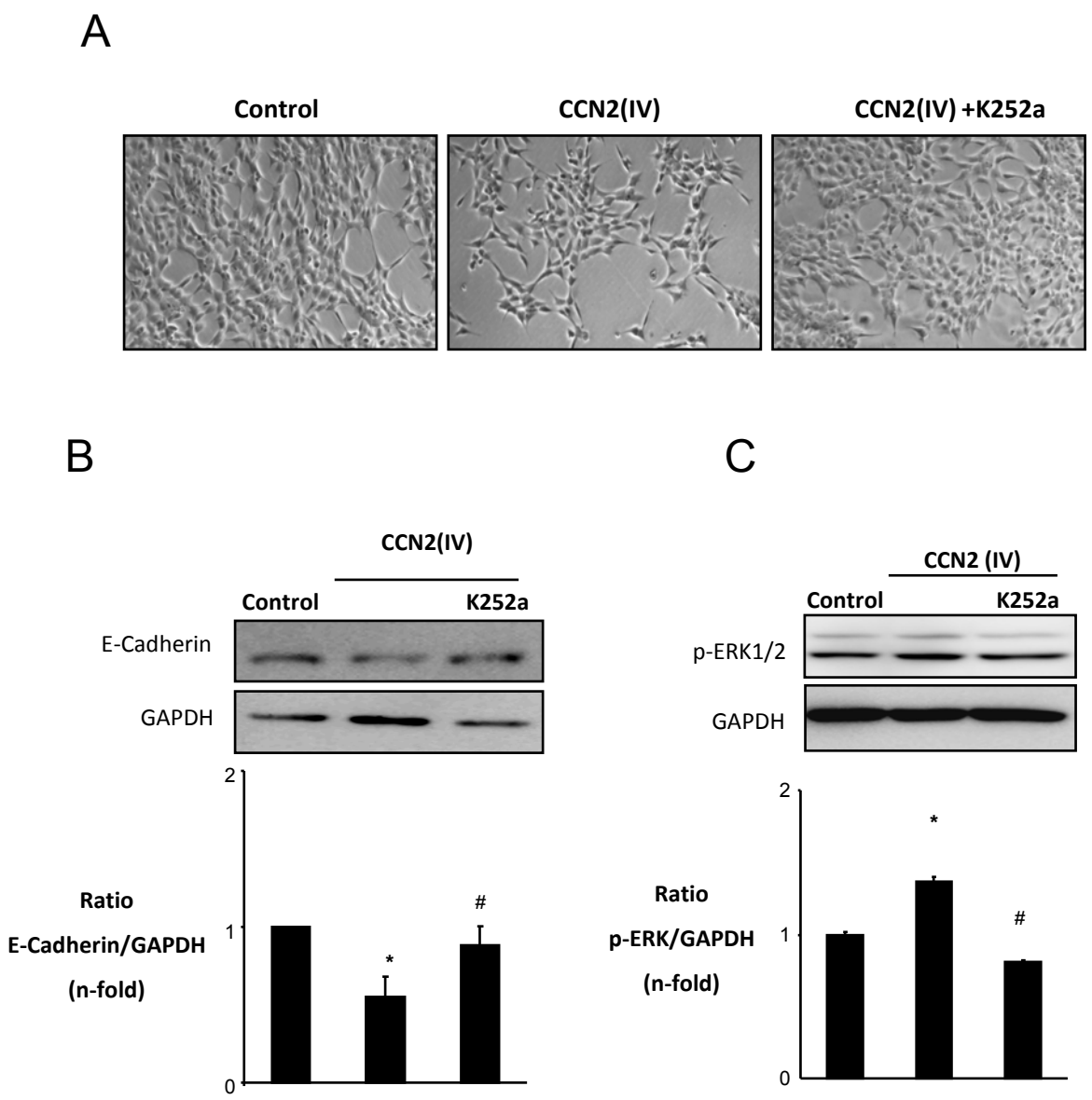

Figure 2. CCN2(IV) via TRkA can promote EMT in MCT cells. Cells were pre-incubated for 1 hour with TrKA inhibitor (K252a) (10 µmol/L). Then, Cells were stimulated with CCN2(IV) (10 ng/mL) for 48 hours. **(A)** Phase contrast microscopy analysis of the cells morphology. **(B)** Changes in E-cadherin protein expression were analyzed by Western blot. Representative autoradiograms are shown. **(C)** Cells were pre-incubated for 1 hour with TrKA inhibitor (K252a) (10 µmol/L). Then, Cells were stimulated with CCN2(IV) (10 ng/mL) for 15 min and p-ERK 1/2 proteins levels were analized by Western blot.Results are data ± SEM from at least 4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone.

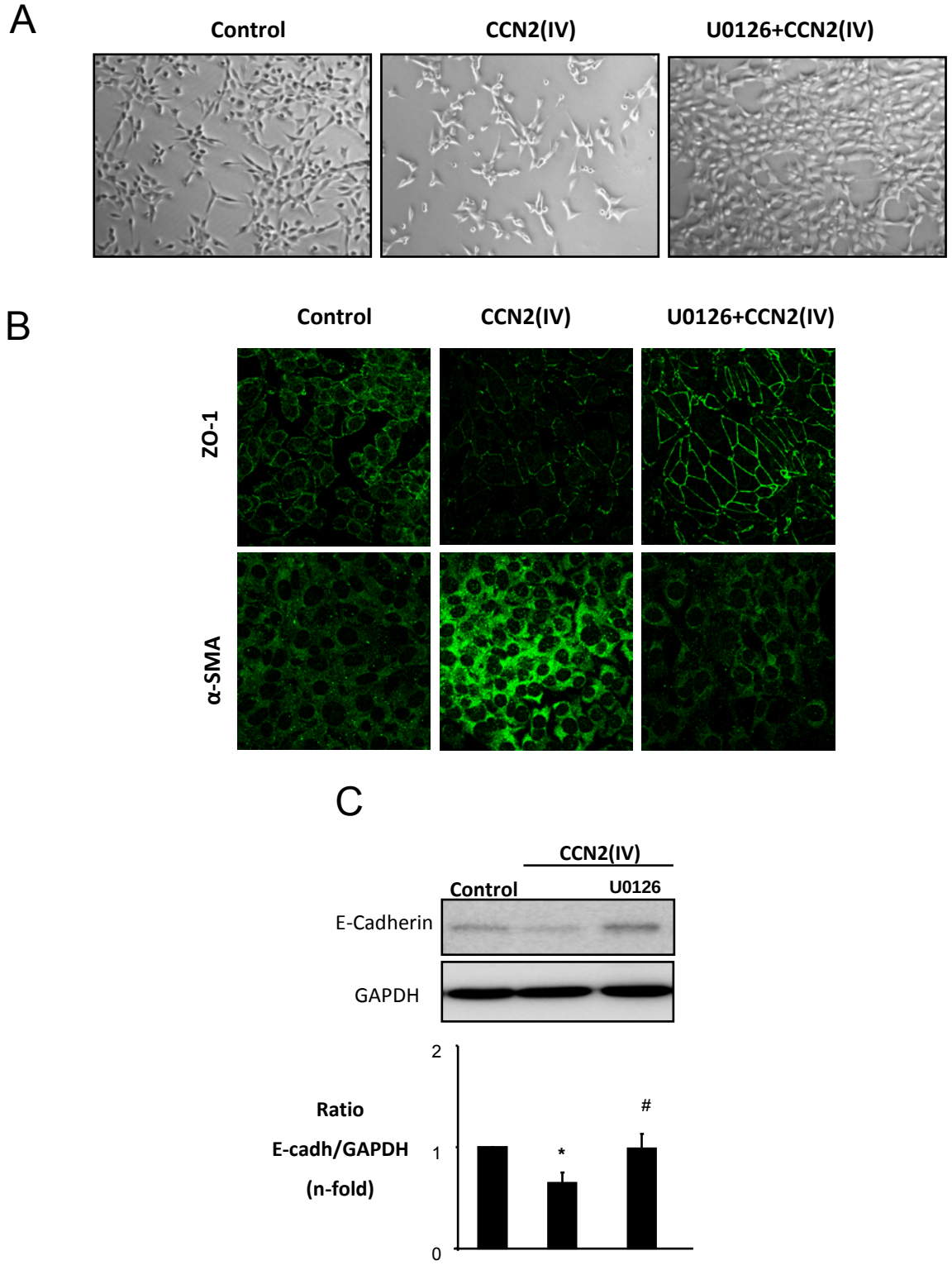

Figure 3. CCN2(IV) via ERK can promote EMT in MCT cells. Cells were pre-incubated for 1 hour with the ERK 1/2 inhibitor U0126 (10 μmol/L).Then, Cells were stimulated with CCN2(IV) (10 ng/mL) for 48 hours. **(A)** Phase contrast microscopy analysis of the cells morphology. **(B)** Confocal microscopy analysis of α-SMA and ZO-1 immunofluorescence, using specific primary antibodies and a FITC-labeled secondary IgG was done. This represents the results of 3 independent observations. **(C)** Changes in E-cadherin protein expression were analyzed by Western blot. Representative autoradiograms are shown. Results are data ± SEM from at least 4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone.

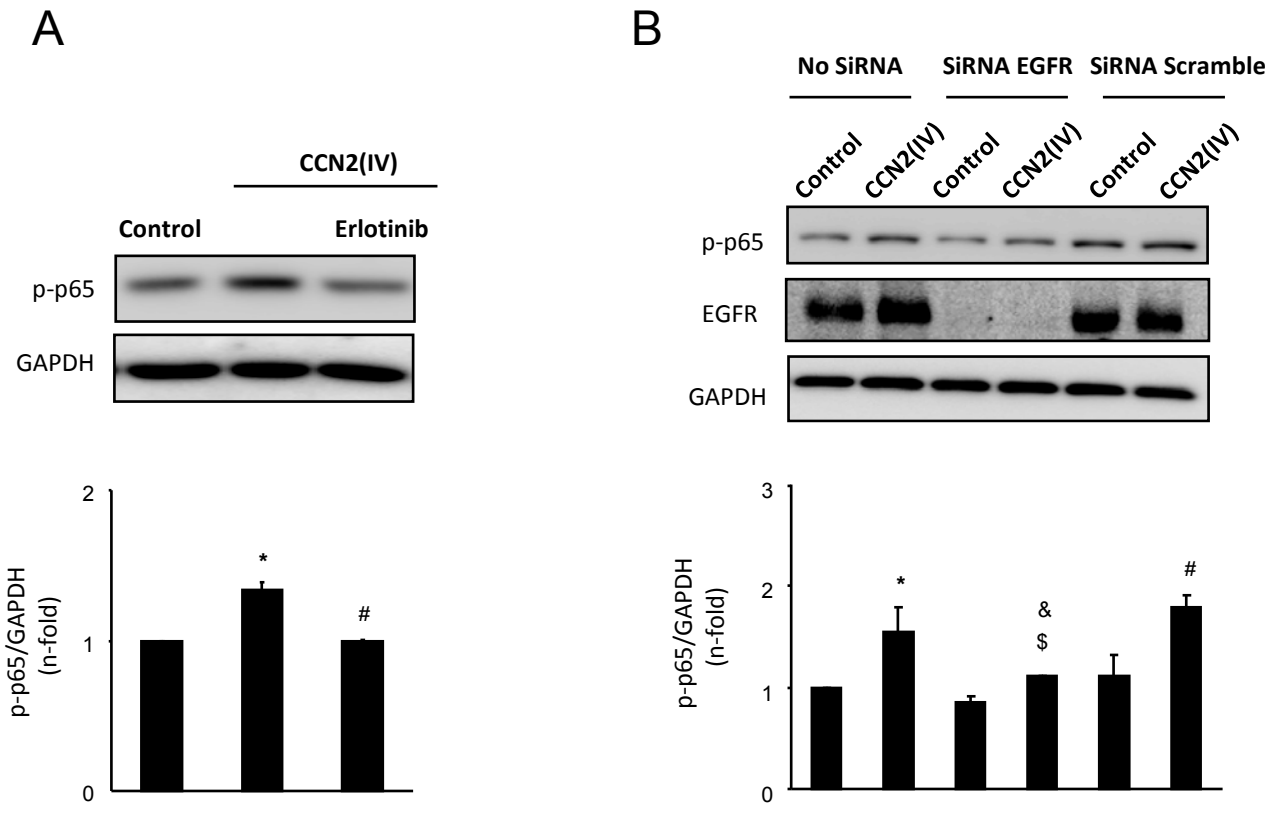

Figure 4. CCN2(IV) via EGFR activates canonical NF-κB pathway. (A) Cells were pre-incubated for 1 hour with the EGFR inhibitor erlotinib (10 μM) and then stimulated with 10 ng/mL CCN2(IV) for 15 min. (B) HK2 cells were transfected with an EGFR siRNA or scrambled siRNA or incubated with transfection reagent alone (non siRNA), as described in "methods". Cells were treated with 10 ng/ml CCN2(IV) for 15 min. p65 phosphorylation was evaluated by Western blot using an antibody against p-p65. GAPDH and EGFR levels were used as loading or silencing control respectively. Figures show a representative Western blot experiment and the quantification expressed as ratio p-p65 vs GAPDH as mean ± SEM of 3 independent experiments. *p<0.05 vs control-untransfected (non siRNA). # p<0.05 vs. untreated scramble siRNA-transfected cells. $ p<0.05 vs. TWEAK-treated scramble siRNA-transfected cells. & p<0.05 vs. TWEAK-treated untransfected cells.

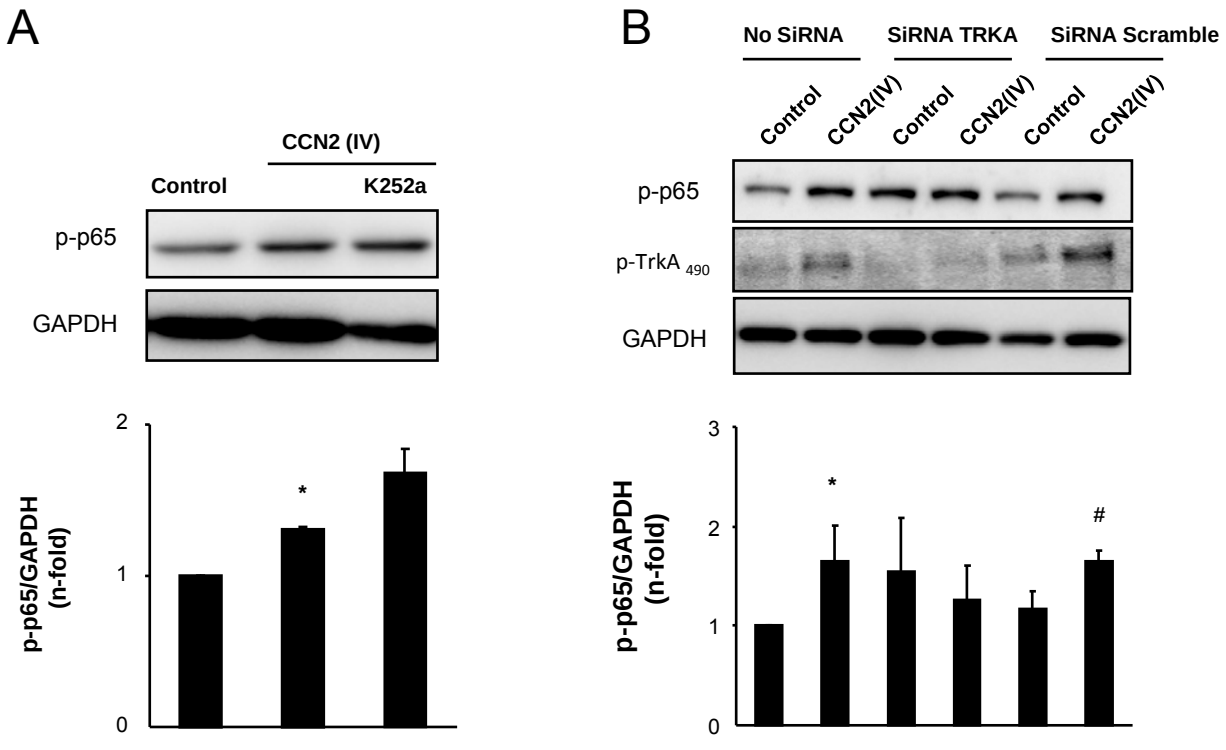

Figure 5. CCN2(IV) activates canonical NF-κB independent of TrKA. (A) Human tubular epithelial (HK2) cells were preincubated for 1 hour with the TrKA inhibitor K252a (10 µmol/L) and then treated or not with 10 ng/mL CCN2(IV) for 15 min. **(B)** HK2 cells were transfected with an TrkA siRNA or scrambled siRNA or incubated with transfection reagent alone (non siRNA). Cells were treated with 10 ng/ml CCN2(IV) for 15 min. p65 phosphorylation was evaluated by Western blot using an antibody against p-p65. GAPDH and p-TrKA levels were used as loading or silencing control respectively. Figures A and B show a representative Western blot experiment and the quantification expressed as ratio p-p65 vs GAPDH as mean ± SEM of 3 independent experiments. *p<0.05 vs control-untransfected (non siRNA). # p<0.05 vs. untreated scramble siRNA-transfected cells.

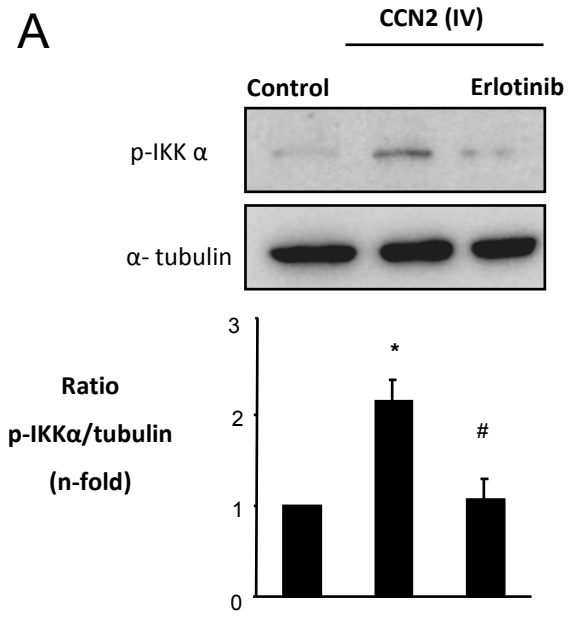

Figure 6. CCN2(IV) via EGFR activates non-canonical NF-κB pathway. Human tubular cells were pretreated with erlotinib 1 hour previous to CCN2(IV) stimulation (10 ng/ml) for 6 hour. Activation of NF-κB2 pathway was determined by evaluation changes IKK-α phosphorylation (A) and RelB nuclear proteins levels (B). Figures show a representative experiment and data as mean ± SEM of 3 independent experiments. *p<0.05 vs control.

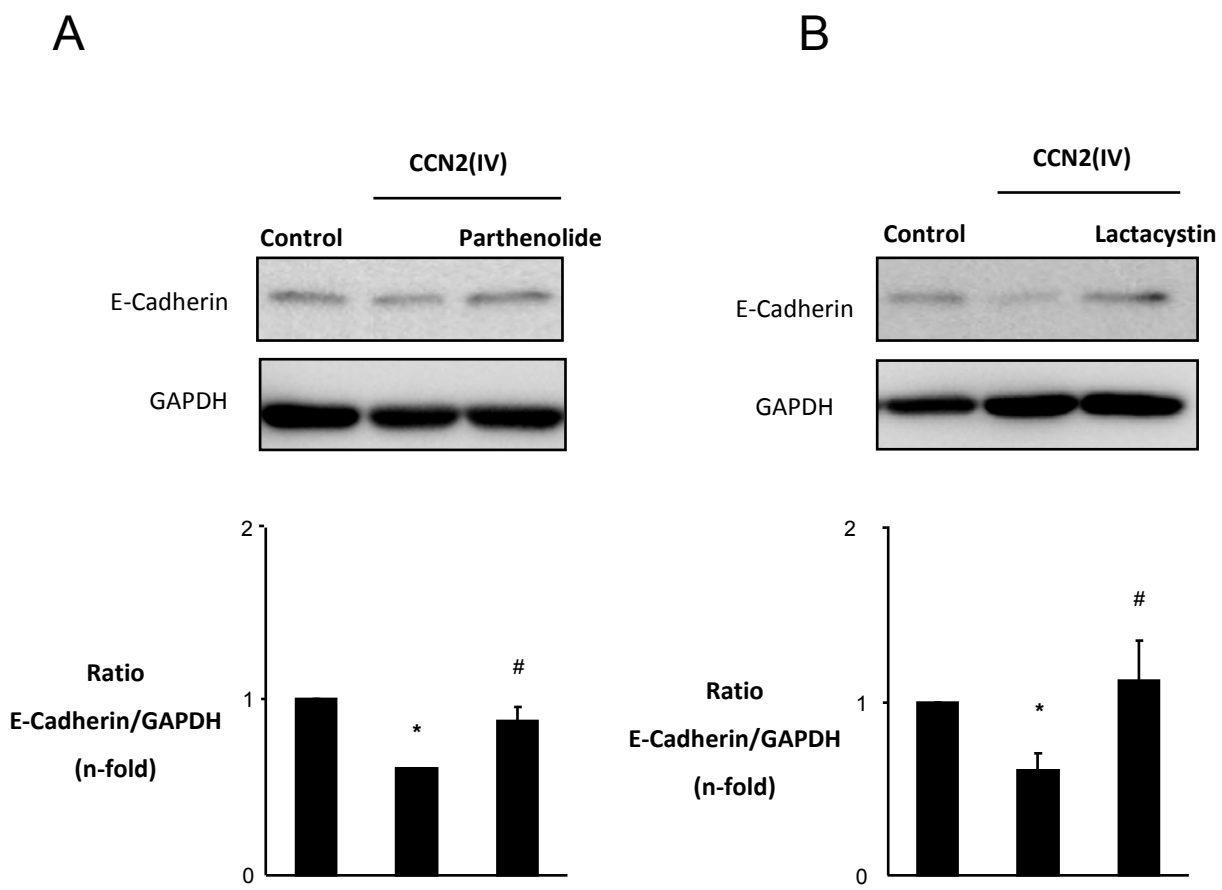

Figure 7. CCN2(IV), via NF-κB activation, induces EMT in cultured tubular epithelial cells. Cells were pre-incubated for 1 hour with NF-κB1 inhibitor (Parthenolide; 1 μmol/L) or the NF-κB2 inhibitor (Lactacystin; 10 μM). Then, Cells were stimulated with 10 ng/mL CCN2(IV) for 48 hours. (A and B) Changes in E-cadherin protein expression were analyzed by Western blot. Representative autoradiograms are shown. Results are data ± SEM from at least 4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone.

METHODS

Cell cultures

MCT cells are a well-characterized line of murine proximal tubular epithelial cells[61]. Cells were counted, seeded and grown in RPMI 1640 medium (GIBCO, Grand Island, NY), supplemented with in the presence of 10% heat-inactivated fetal bovine serum (FBS). Then cells were cultured at 37ºC in 5% CO2 atmosphere.

HK-2 cells (immortalized human tubuloepithelial cell line) were grown in RPMI 1640 medium with 10% of FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, (Sigma) and Hyidrocortisone (36ng/mL) (Sigma) at 37ºC in 5% CO2 atmosphere. At confluence, cells were serum deprived for 24 hours and then experiments were done.

Renal tubuloepithelial mouse cortical tubule (MCT) cells and the human proximal tubule cell line HK-2, were grown in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS), supplemented with ITS (5 μg/mL), 1% and hydrocortisone (36 ng/ml) (HK-2 cells), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in 5% CO_2 at 37° C. At confluence cells were made quiescent for 24 hours and then different studies were performed. Subconfluent cells (60,000 cells/cm^2) were incubated with the stimuli in this medium without (HK-2) or with 1% FBS (MCT) for different times.. In some experiments, the following inhibitors were added 1 h before CCN2(IV): Erlotinib (100 nmol/L) from Vichem, tyrphostin AG 1478 (100 nmol/L) (Alomone Labs), U0126 (10 μmol/L) (Promega); Lactacistine (10 μmol/L) (Sigma); Parthenolide (10 μmol/L) (Sigma).

Protein studies

Proteins were obtained from treated cells or mouse kidneys using lysis buffer (50 mmol/L Tris-HCl, 150 mol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% Triton X-100, 0.3% IGEPAL, 10 μl/ml proteinase inhibitors cocktail, 0.2 mmol/L PMSF, and 0.2 mmol/L orthovanadate). To determine protein content the BCA method was used.

For western blot, cell (25 μg/lane) protein extracts were separated on 6%-12% polyacrylamide-SDS gels under reducing conditions. Samples were then transferred onto nitrocellulose membranes (BioRad), blocked with TBS/5% defatted milk/0.05% Tween-20, and incubated overnight at 4ºC with the following antibodies (dilution): anti-ecadherin (1:500; BD), anti- p100/p52 [1:200], p-p65 [1:200] (Cell Signalling); p-ERK1/2 [1:200]; rel B and p-IKKα [1:200] (Santa Cruz Biotechnology). Membranes were subsequently incubated with peroxidase-conjugated IgG secondary antibody and developed using an ECL chemiluminiscence kit (Amersham). Loading controls were done using an anti-GAPDH antibody (1:10000; Chemicon), anti-α tubulin (1:5000; Sigma), or red ponceau for nuclear proteins. Autoradiographs were scanned using the Gel Doc™ EZ imager and analyzed with the Image Lab 3.0 software (BioRad).

Gene silencing

Gene silencing in cultured cells was performed using either pre-designed siRNA corresponding to EGFR and TrKA, or their corresponding scramble siRNAs (Ambion). Subconfluent cells were transfected for 24 h with 25nmol/L siRNA using 50 nmol/L Lipofectamine RNAiMAX (Invitrogen) or treated only with lipofectamine-vehicle, according to the manufacturer's instructions. Then, cells were incubated with 10% heat-inactivated FBS for 24 h, followed by 24 h in serum-free medium before the experiments.

Immunofluorescence for EMT markers

Cells grown on coverslips were stimulated with the agonists, and then fixed in Merckofix (Merck, Whitehouse Station, NJ, USA) and permeabilized with 0.1% Triton-X100 for 2 min. After blocking with 10% BSA and 10% FBS for 1 h, they were incubated with several primary antibodies [dilution, -fold]: rabbit polyclonal anti-Z0-1 [200] (Zymed Laboratories. San Francisco, CA); anti-α-SMA [200] (ABCam, Cambridge, MA) [200]; and anti-snail (Sta. Cruz Biotechnology) [200] antibodies for 1 h, followed by a fluorescein isothiocyanate (FITC)-conjugated secondary antibody [200], for 1 h. Absence of primary antibody was used as negative control. Samples were mounted in Mowiol 40-88 (Sigma-Aldrich) and examined by a Leica DM-IRB confocal microscope.

Statistical analysis

Results throughout the text are expressed as mean±SEM. Differences between agonist-treated groups and controls were assessed by one-way analysis of variance, followed by post-hoc Bonferroni or Dunnett test, or Mann-Whitney test, as appropriate. $P<0.05$ was considered significant. Statistical analysis was conducted using the SPSS statistical software (version 11.0, Chicago, IL).

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3. La proteína relacionada con la Paratohormona regula la Transición epitelio mesénquimal mediante la interacción con TGF-β y EGF en células tubuloepiteliales renales.

La fibrosis túbulointesticial es una característica común a patologías renales crónicas. Se ha descrito que la Transición Epitelio-Mesenquimal (TEM) es un mecanismo que contribuye a la fibrosis renal al inducir la conversión de las células tubuloepiteliales a miofibroblastos productores de matriz extracelular (Iwano et al., 2002, Zeisberg et al., 2008). Datos recientes sugieren que la PTHrP podría participar en la fibrosis renal (Ardura et al., 2008). En esta parte del estudio, que se corresponde con el objetivo 2, se describe que en células tubuloepiteliales de ratón, PTHrP(1-36) induce TEM, caracterizada por cambios en el fenotipo celular de carácter epitelial hacia miofibroblástico, pérdida de marcadores epiteliales así como inducción de marcadores mesenquimales. Un mecanismo clave en la TEM podría ser la activación de la ruta del EGFR, pero se desconoce si PTHrP(1-36) puede activar esta vía de señalización. En este trabajo se demuestra como la PTHrP *in vivo* contribuye a la activación de la ruta del EGFR. En un modelo de daño renal por obstrucción unilateral del uréter, en ratones que sobreexpresan PTHrP en el túbulo, se observó un mayor aumento de la fosforilación del EGFR en los ratones obstruidos en comparación con los riñones contralaterales, efecto asociado a su vez con cambios en marcadores de TEM. A su vez PTHrP(1-36) es capaz de transactivar el EGFR en células tubuloepiteliales renales, a través de un proceso regulado por 2 mecanismos: activación de MMPs vía PKC y activación mediada por Src. El bloqueo de la transactivación del EGFR, mediante un inhibidor de la quinasa del EGFR, revirtió los cambios asociados a la TEM inducidos por la exposición a PTHrP(1-36). Entre las señales intracelulares implicadas en la regulación de la TEM destaca la activación de la quinasa ERK (Grotegut et al., 2006; Santibanez, 2006; Bhowmick et al., 2001). El tratamiento con inhibidores de ERK (U0126 y PD98059) bloqueó la TEM causada por PTHrP(1-36). Estos datos muestran que PTHrP(1-36) interactúa con EGFR para inducir TEM a través de un mecanismo que converge en la activación de ERK en células tubuloepiteliales renales.

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Parathyroid Hormone–Related Protein Promotes Epithelial–Mesenchymal Transition

Juan Antonio Ardura,* Sandra Rayego-Mateos,† David Rámila,* Marta Ruiz-Ortega,† and Pedro Esbrit*

*Bone and Mineral Metabolism Laboratory and †Cellular Biology in Renal Diseases Laboratory, Fundación Jiménez Díaz (Capio Group) and Universidad Autónoma de Madrid, Madrid, Spain

ABSTRACT

Epithelial–mesenchymal transition (EMT) is an important process that contributes to renal fibrogenesis. TGF-β1 and EGF stimulate EMT. Recent studies suggested that parathyroid hormone–related protein (PTHrP) promotes fibrogenesis in the damaged kidney, apparently dependent on its interaction with vascular endothelial growth factor (VEGF), but whether it also interacts with TGF-β and EGF to modulate EMT is unknown. Here, PTHrP(1-36) increased TGF-β1 in cultured tubuloepithelial cells and TGF-β blockade inhibited PTHrP-induced EMT-related changes, including upregulation of α-smooth muscle actin and integrin-linked kinase, nuclear translocation of Snail, and downregulation of E-cadherin and zonula occludens-1. PTHrP(1-36) also induced EGF receptor (EGFR) activation; inhibition of protein kinase C and metalloproteases abrogated this activation. Inhibition of EGFR activation abolished these EMT-related changes, the activation of ERK1/2, and upregulation of TGF-β1 and VEGF by PTHrP(1-36). Moreover, inhibition of ERK1/2 blocked EMT induced by either PTHrP(1-36), TGF-β1, EGF, or VEGF. *In vivo*, obstruction of mouse kidneys led to changes consistent with EMT and upregulation of TGF-β1 mRNA, p-EGFR protein, and PTHrP. Taken together, these data suggest that PTHrP, TGF-β, EGF, and VEGF might cooperate through activation of ERK1/2 to induce EMT in renal tubuloepithelial cells.

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In the injured kidney, interstitial fibroblasts are considered the main cell type responsible for fibrogenesis. During this process, these cells proliferate and can become activated to myofibroblasts, identified by *de novo* expression of α-smooth muscle actin (α-SMA), a prognostic marker for the progression of fibrogenesis.[1,2] It is currently known that a large proportion of interstitial myofibroblasts in fibrotic kidneys originate from tubuloepithelial cells through epithelial to mesenchymal transition (EMT).[2,3] On the other hand, EMT may also contribute to the repair process of the damaged tubules. Thus, after renal injury, remnant tubuloepithelial cells dedifferentiate to mesenchymal cells, which migrate toward the damaged areas, where they proliferate and subsequently differentiate into the original epithelial phenotype to restore tubular integrity.[4]

EMT is a multistep process that requires the integration of multiple extrinsic and intrinsic pathways. Epithelia in transition lose polarity, adherence, and tight junctions and rearrange the F-actin cytoskeleton, associated with upregulation of many genes used as EMT markers.[5] The latter includes, in addition to α-SMA, which increases cell contractility and motility,[6] extracellular matrix proteins such as fibronectin and several types of collagens,[3,5] metalloproteases (MMPs) 2 and 9 implicated in the

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Correspondence: Dr. Pedro Esbrit, Laboratorio de Metabolismo Mineral y Óseo, Fundación Jiménez Díaz-UTE, Avda. Reyes Católicos 2, 28040 Madrid, Spain. Phone: 34-91-550-4894; Fax: 34-91-549-8075; E-mail: pesbrit@fjd.es

basal layer degradation,[7,8] and integrin linked kinase (ILK), present in focal contacts necessary to cell movement.[9] In addition, a decrease in the expression of proteins that keep basolateral polarity, namely cytokeratin, and intercellular junctions, including the adherent junction proteins E-cadherin and β-catenin, takes place in the renal tubuloepithelium during EMT.[5] Activation of different signaling pathways act as EMT intrinsic regulators: mitogen activated kinases (MAPKs), namely extracellular signal-regulated kinases (ERKs)1/2, p38, and N-terminal c-Jun kinase (JNK); the Ras protein family (Ras, Rho, and Rac); Wnt and Smad proteins; and the transcription factors snail and slug.[5,10–12]

Different studies support the important role of various profibrogenic factors on the EMT process in the kidney.[8,13–16] The intracellular mechanisms underlying EMT induction by these factors are poorly known but at least in the case of TGF-β, the main factor studied in this regard, they seem to involve activation of MAPKs and Smad proteins.[12,16,17] In addition, activation of EGF receptor (EGFR) tyrosine kinase can trigger various cell responses including EMT in tubular cells, at least in part through MAPKs signaling pathway.[14,17,18] Moreover, induction of EMT by oxidative and osmotic stress and hypoxia—conditions associated with renal injury—can be accounted for in part by EGFR transactivation.[19]

Parathyroid hormone (PTH)-related protein (PTHrP) is upregulated in various experimental nephropathies.[20] In addition, a preliminary report has shown that PTHrP upregulation occurs in both tubules and glomeruli in patients with diabetic nephropathy.[21] Recent studies in mice indicate that PTHrP can act as a proinflammatory and profibrogenic factor in the acutely damaged kidney after folic acid injection or unilateral ureteral obstruction.[22–24] Moreover, one of these studies strongly suggests that the profibrogenic action of PTHrP might be accounted for in part by promoting EMT through interaction with vascular endothelial growth factor (VEGF).[24] Interestingly in this scenario, it has previously been suggested that TGF-β might act as a modulator of at least some PTHrP actions through the PTH receptor 1 (PTHR1), common to PTH and PTHrP, in its target cells, such as tubuloepithelial cells and osteoblasts. Hence, TGF-β has shown to attenuate the inhibition of phosphate transport elicited by activation of this receptor in proximal tubule cells,[25] whereas PTH itself can induce the expression of TGF-β and amplifies its stimulatory effect on type I collagen production in osteoblastic cells.[26,27] Moreover, a recent report has shown that PTHR1 overexpression in a human osteosarcoma cell line resulted in its increased invasive capacity, associated with upregulation of TGF-β1.[28] Also of interest in the present context, in both human embryonic kidney cells HEK-293 and osteoblasts, stimulation of PTHR1 may lead to EGFR transactivation.[29,30] Collectively, these findings suggest that PTHrP might interact with TGF-β and/or EGFR to modulate EMT. To confirm this hypothesis, in this study, we aimed to characterize such interactions and their relative contribution to the mechanisms whereby PTHrP may induce EMT in renal tubuloepithelial cells.

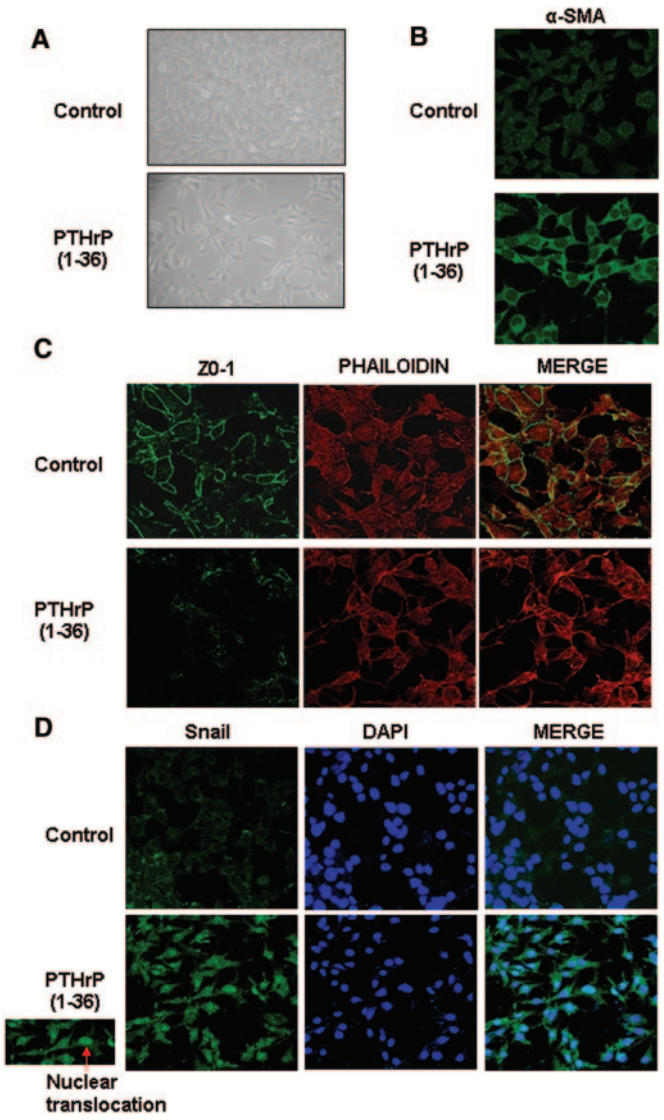

Figure 1. PTHrP(1-36) causes EMT in renal tubuloepithelial mouse cortical tubule (MCT) cells. Cells were stimulated with or without (control) PTHrP(1-36) (100 nM) for 48 h in culture medium. (A) Representative phase-contrast images of control and PTHrP(1-36)–stimulated MCT cells are shown (original magnification, ×200). Confocal microscopy analysis of α-SMA (B), ZO-1 (C), and snail (D) by immunofluorescence was performed using specific primary antibodies and a FITC-labeled secondary IgG. (C) TRITC-conjugated phalloidin (red staining) was used as a cytoskeleton marker to depict cell morphology. (D) Double immunofluorescence staining was assessed, using 4′,6-diamino-2-phenylindole dihydrochloride for the nucleus (blue) and FITC-labeled IgG (green) for snail. The inset shows in detail the presence of a green image in the cell nucleus. The overlaid images in green and blue (merge) yielded a white tone in the nucleus, indicating snail nuclear localization. This represents the results of three independent observations.

RESULTS

PTHrP(1-36) Can Induce EMT Through TGF-β in Mouse Cortical Tubule Cells

Incubation with PTHrP(1-36), at 100 nM, for 48 h causes a conversion of mouse cortical tubule (MCT) cells from an epithelial to a myofibroblast-like phenotype. As depicted by light microscopy, these transdifferentiated cells lost the typical cobblestone pattern of an epithelial monolayer and displayed a spindle-shape, fibroblast-like morphology (Figure 1A). These changes were associated with induction of the mesenchymal marker α-SMA and a decrease in the epithelial marker zonula occludens-1 (ZO-1) in these cells, as shown by confocal microscopy (Figure 1, B and C). Phalloidin staining, which detects F-actin, visualizes the cytoskeleton, showing actin fibers rearrangement (Figure 1C). In addition, PTHrP(1-36) increased snail immunostaining and induced its nuclear translocation (Figure 1D), where it can act as an E-cadherin transcription repressor. Snail also downregulates other epithelial markers but upregulates some mesenchymal markers (namely fibronectin and vitronectin) during EMT.[31,32] These findings further support and extend recent data,[22,24] indicating that PTHrP(1-36) promotes EMT in MCT cells.

As mentioned above, TGF-β is one of the main inducers of EMT.[13–17] We tested here whether TGF-β could be a downstream mediator of PTHrP(1-36)–induced EMT in MCT cells. First, we evaluated whether this peptide would affect TGF-β production by these cells in a similar manner to PTH in osteoblastic cells.[26] We found that TGF-β1 mRNA was induced by PTHrP(1-36), at 100 nM, as early as 3 h, and it remained increased up to at least 18 h in MCT cells (Figure 2A). To establish whether the PTHrP(1-36)–induced rise in TGF-β1 mRNA was accompanied by TGF-β1 protein synthesis, its levels were measured in the MCT-conditioned medium. As expected,[33] this factor was mainly secreted as an inactive protein by these cells. In contrast, an increase in TGF-β1 secretion, which consisted of its active form, was elicited by PTHrP(1-36) at 48 h (Figure 2B).

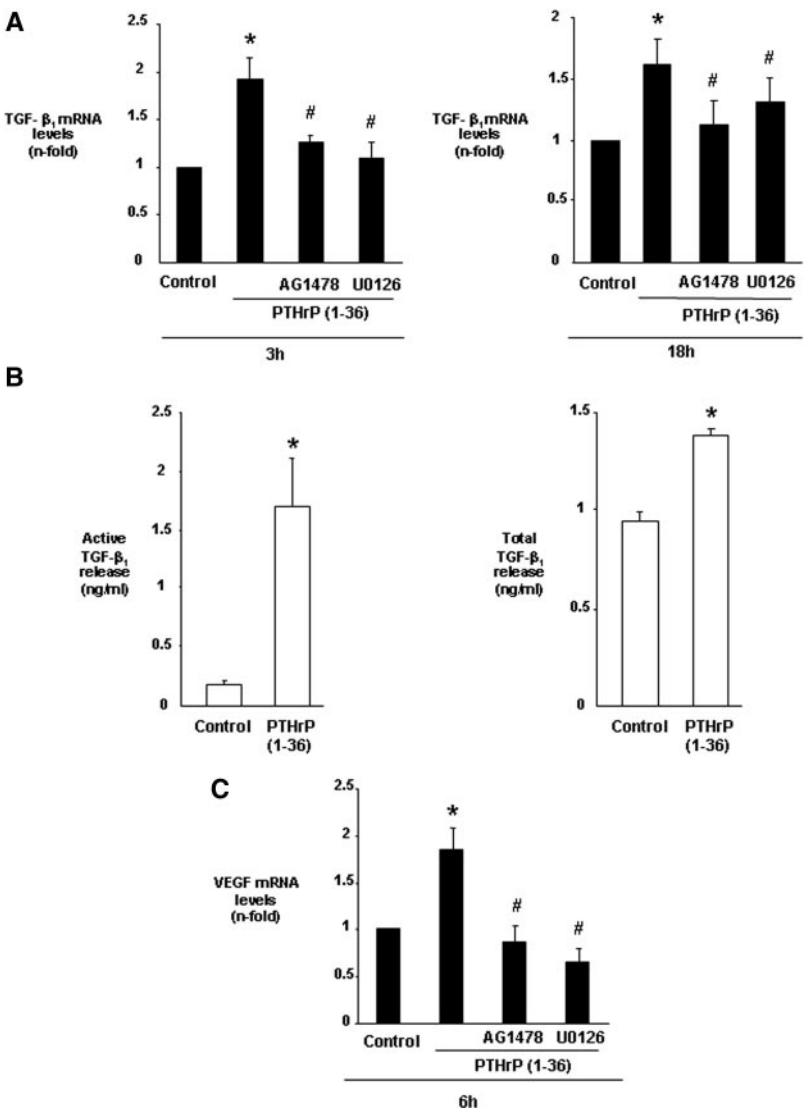

Figure 2. Early induction of both TGF-β_1 and VEGF by PTHrP(1-36) in MCT cells. MCT cells were stimulated with PTHrP(1-36) (100 nM) for different time periods. In some experiments, cells were pretreated for 1 h with an EGFR inhibitor (tyrphostin AG 1478, 100 nM) or an ERK1/2 inhibitor (U0126, 10 μM) before addition of PTHrP(1-36). Total cell RNA was isolated to assess mRNA levels of TGF-β_1 (A) and VEGF (C) by real-time PCR. (B) After PTHrP(1-36) stimulation for 48 h, total and active (after transient acidification) TGF-β_1 was measured in the cell-conditioned medium by a specific ELISA. Data are expressed as mean ± SEM of four independent experiments. *$P < 0.05$ *versus* control; #$P < 0.05$ *versus* PTHrP(1-36) alone.

We next used different strategies to block TGF-β at the time of MCT stimulation with PTHrP(1-36) by adding a neutralizing antibody against active TGF-β, which has proven to block angiotensin II–induced extracellular matrix production and EMT,[33,34] an inhibitor of the TGF-β type I receptor (TRI) kinase (TRI-ki), which prevents TGF-β–induced responses through the inactivation of TRI kinase,[34] and decorin, a proteoglycan that inhibits active TGF-β, thus acting as an antagonist.[35] All of these TGF-β blockers were shown to antagonize several EMT-related changes induced by PTHrP(1-36), as shown by immunofluorescence, in MCT cells (Figure 3A). Moreover, by Western analysis, E-cadherin was downregulated by this PTHrP peptide in these cells, consistent with recent findings,[24] an effect that was also abrogated by all these TGF-β blockers (Figure 3B). These data strongly suggest that this factor may act as a mediator of EMT induction by PTHrP(1-36) in tubuloepithelial cells.

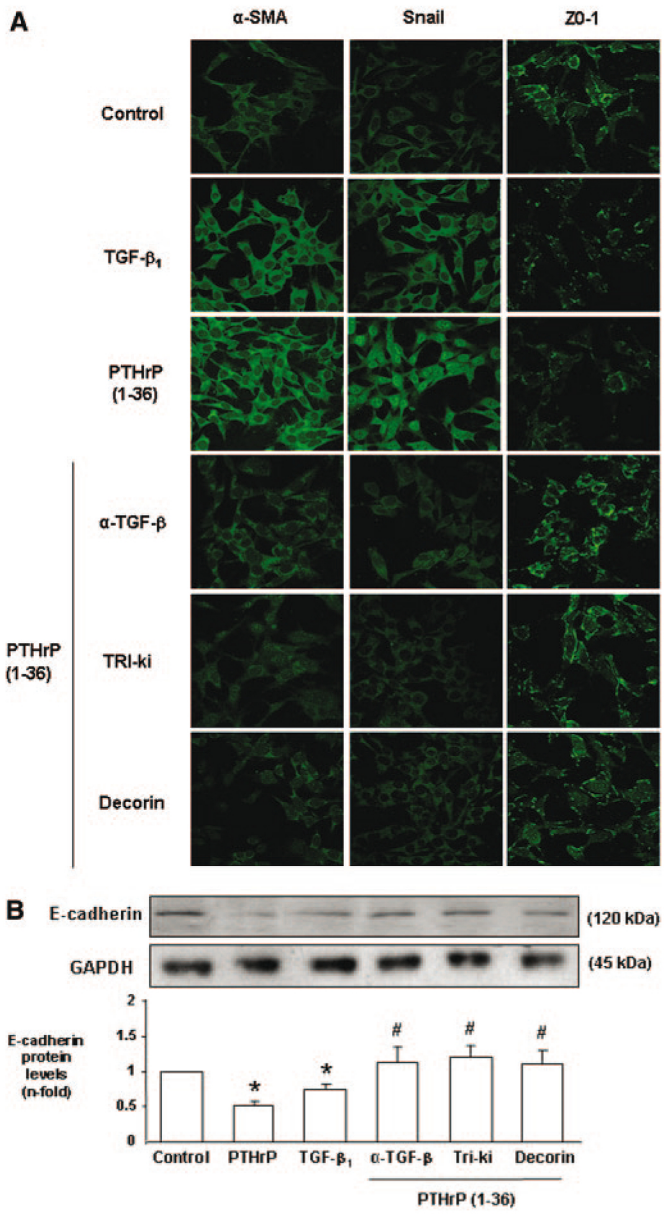

Figure 3. TGF-β acts as a mediator of PTHrP(1-36)–induced EMT in MCT cells. TGF-β was blocked or not (control) by pretreatment of MCT cells for 1 h with a TGF-β neutralizing antibody (α-TGF-β; 10 μg/ml), TRI-ki (10 μM), and the antagonist decorin (100 nM). Cells were stimulated with PTHrP(1-36) (100 nM) for 48 h. TGF-β1 (1 ng/ml) was used as positive control. (A) Detection of α-SMA, snail, and ZO-1 was performed by indirect immunofluorescence using FITC-labeled secondary IgG and confocal microscopy. This represents the results of three independent observations. (B) After PTHrP(1-36) stimulation, total cell protein was isolated to evaluate E-cadherin protein expression by Western blot. A representative autoradiogram is shown (top panel). GAPDH was used as loading control. Experimental values are mean ± SEM from six independent experiments and were expressed as n-fold over control. *$P < 0.05$ *versus* control; #$P < 0.05$ *versus* PTHrP(1-36) alone.

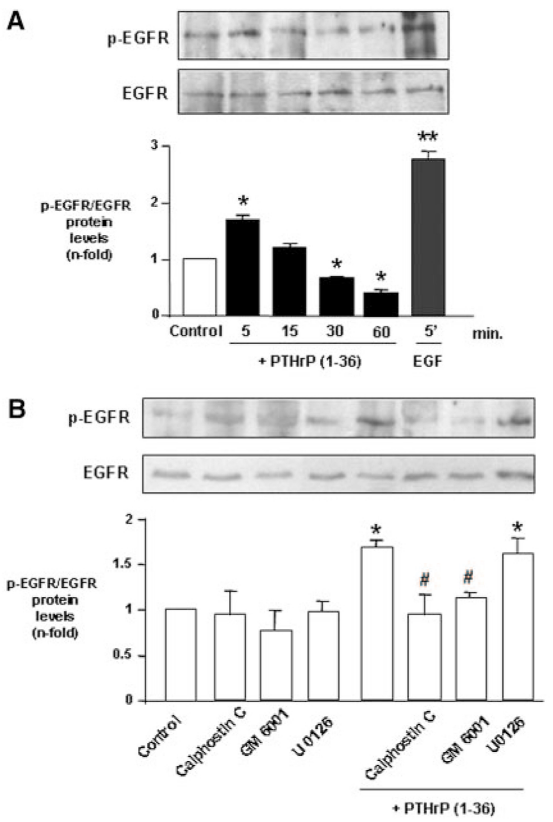

Figure 4. PTHrP(1-36) induces EGFR phosphorylation in MCT cells. Cells were stimulated with PTHrP(1-36) (100 nM) for different time periods (A) or for 5 min (B). EGFR phosphorylation was assesed by EGFR immunoprecipitation and Western analysis with p-tyrosine PY20 antibody as described in the Concise Methods. Total EGFR protein levels were used as phosphorylation control. (B) The following agents were added 1 h before PTHrP(1-36) stimulation: a PKC inhibitor (calphostin C, 250 nM), a pan-specific MMPs inhibitor (GM 6001, 1 μM), and an ERK 1/2 inhibitor (U0126, 10 μM). Values are mean ± SEM from at least three independent experiments by triplicate. *$P < 0.05$ and **$P < 0.01$ *versus* control. #$P < 0.05$ *versus* PTHrP(1-36) alone.

PTHrP(1-36) Induces EMT-Related Changes by EGFR Transactivation in MCT Cells

Congruent with previous data in an embryonic kidney cell line,[30] we found here that PTHrP(1-36), at 100 nM, rapidly (within 5 min) and transiently induced EGFR phosphorylation in MCT cells (Figure 4A). A major mechanism for EGFR transactivation by ligands interacting with G protein–coupled receptors (GPCRs), including PTH1R, involves activation of Gq/PKC (but not G_s) and proteolytic processing of EGFR ligands by MMPs.[29,30] Consistent with this notion, both calphostin C, a protein kinase C inhibitor, and GM6001, a pan-specific inhibitor of metalloproteases, were found to abolish EGFR activation by PTHrP(1-36) in MCT cells (Figure 4B).

We evaluated whether EGFR transactivation could also be a mediator of the PTHrP(1-36)–induced EMT in MCT cells. Cell preincubation with tyrphostin AG1478, a specific EGFR inhibitor, blocked the changes elicited by this PTHrP peptide

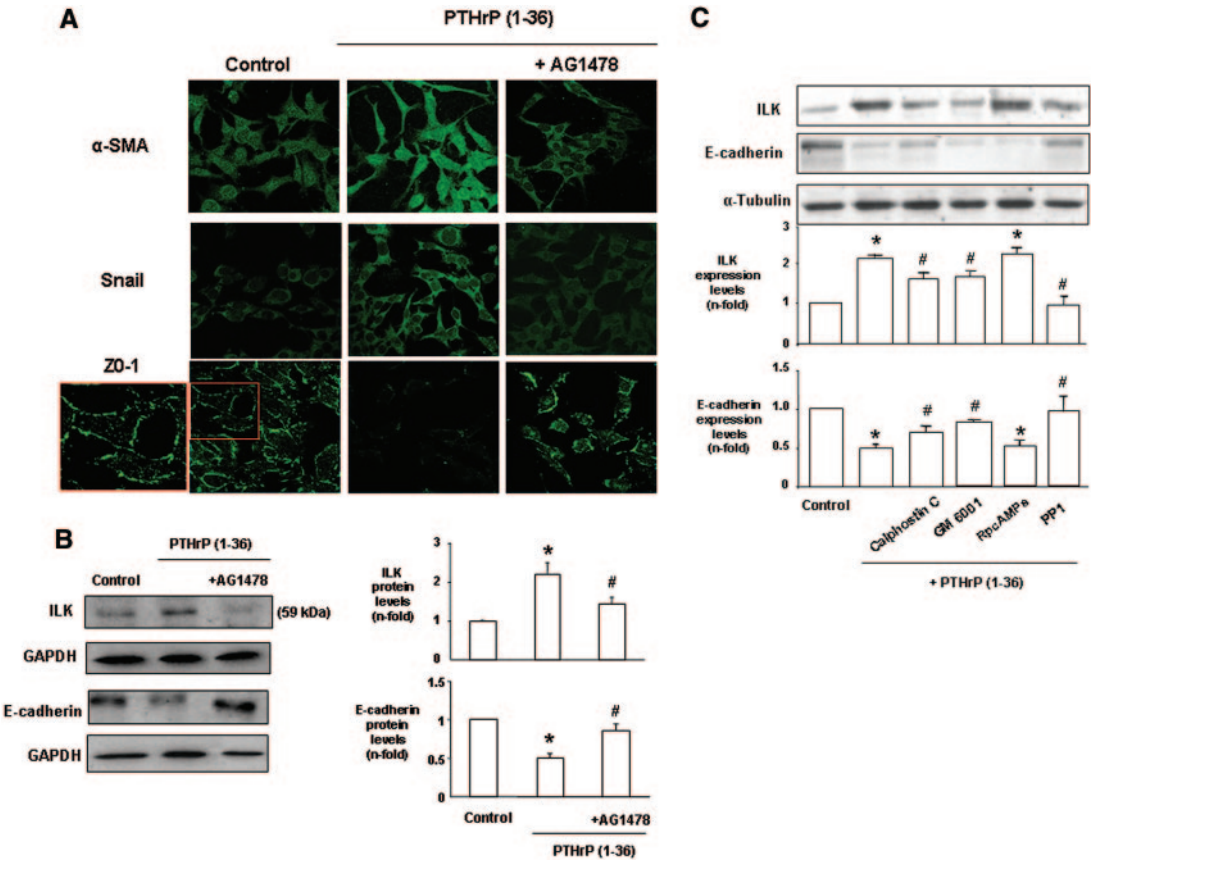

Figure 5. PTHrP can promote EMT by inducing EGFR transactivation in MCT cells. Cells were preincubated for 1 h with different inhibitors: tyrphostin AG1478 (100 nM), calphostin C (250 nM), GM 6001 (1 μM), PP1 (1 μM), and Rp-cAMPS (1 μM). Cells were stimulated with PTHrP(1-36) (100 nM) for 48 h. (A) Confocal microscopy analysis of α-SMA, snail, and ZO-1 immunofluorescence, using specific primary antibodies and a FITC-labeled secondary IgG was done. The inset shows in detail the presence of a green image denoting the tight junctions in nonstimulated tubuloepithelial MCT cells. This represents the results of three independent observations. (B and C) Changes in ILK and E-cadherin protein expression were analyzed by Western blot. Representative autoradiograms are shown. Results are data ± SEM from at least three independent experiments in triplicate. *$P < 0.05$ *versus* control; #$P < 0.05$ *versus* PTHrP(1-36) alone.

on the expression of several EMT-related proteins, as assessed by immunofluorescence and Western blot (Figure 5, A and B). Supporting further that EGFR transactivation seems to be a mechanism whereby PTHrP(1-36) can induce EMT in MCT cells, both calphostin C and GM6001 significantly decreased the alterations triggered by this PTHrP peptide on ILK and E-cadherin protein expression in these cells (Figure 5C). On the other hand, Rp-cAMPS, a PKA inhibitor, was inefficient in this regard (Figure 5C).

PTHrP Overexpression in the Mouse Obstructed Kidney Undergoing EMT Is Related to Increased TGF-β_1 Expression and EGFR Activation

We recently reported that some EMT-related changes occur associated with PTHrP overexpression in the mouse obstructed kidney.[24] Consistent with the aforementioned *in vitro* findings, we found here that both TGF-β_1 mRNA and p-EGFR protein levels were augmented in the mouse kidney in this *in vivo* scenario (Figure 6, A and B); associated with an increase of α-SMA and snail gene expression (Figure 6C). Moreover, all of these factors tested were consistently upregulated in the unobstructed kidney from transgenic mice with targeted overexpression of PTHrP to the renal proximal tubule, congruent with our recent observations.[24]

Role of ERKs on the Intracellular Mechanism Underlying PTHrP(1-36) Induction of EMT in MCT Cells

The putative involvement of ERK activation on EMT induction by PTHrP(1-36) in tubuloepithelial cells was next studied. After PTHR1 activation, β-arrestin–dependent PTH1R internalization and/or G_q signaling can transactivate EGFR, leading to ERK1/2 phosphorylation.[30] Consistent with this notion, PTHrP(1-36), at 100 nM, was shown to induce ERK1/2 phosphorylation in a manner reminiscent to that observed for EGFR activation in MCT cells.[23] Moreover, the ERK1/2 phosphorylation inhibitor U0126 failed to affect PTHrP(1-36)–induced transactivation of EGFR in these cells (Figure 4B). On the other hand, tyrphostin AG1478, calphostin C, or several

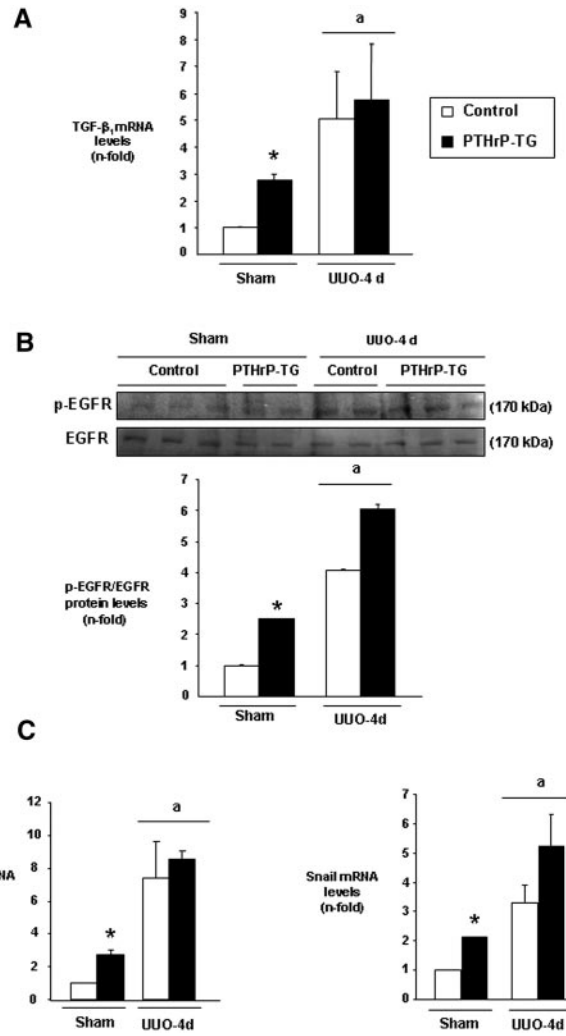

Figure 6. TGF-β_1 mRNA and p-EGFR protein expression associated with EMT-related changes occurs in the obstructed kidney from PTHrP transgenic (PTHrP-TG) mice and their control littermates. On day 4 after UUO, we evaluated gene expression of TGF-β_1 (A) and that of α-SMA and snail (by real-time PCR) (C) and p-EGFR phosphorylation (by Western blot, using antibodies against p-EGFR and EGFR). Representative autoradiograms are shown (B). Experimental values are mean ± SEM of three to four mice per group. All values were normalized against corresponding sham control. *$P < 0.05$ *versus* corresponding value in control mice; [a]$P < 0.05$ *versus* corresponding value in sham-operated mice.

Figure 7. PTHrP(1-36) phosphorylates ERK1/2 by a mechanism depending on EGFR activation in MCT cells. Before PTHrP(1-36) stimulation for 10 min, cells were preincubated for 1 h with different inhibitors: tyrphostin AG1478 (100 nM), calphostin C (250 nM), GM 6001 (1 μM), MMP-2 and 9 inhibitor (1 μM), PP1 (1 μM), and Rp-cAMPS (1 μM). ERK 1/2 phosphorylation was assessed by Western analysis. Total ERK levels were used for control of phosphorylation changes. Values are means ± SEM from at least three independent experiments in triplicate. *$P < 0.05$ *versus* control; #$P < 0.05$ *versus* PTHrP(1-36) alone.

MMP inhibitors (GM6001 and an inhibitor of MMP-2 and MMP-9), in contrast to Rp-cAMPS, significantly inhibited ERK1/2 activation (Figure 7, A and B).

We found that two ERK1/2 activation inhibitors, PD98059 and U0126, prevented PTHrP(1-36)–induced conversion of the epithelial to a myofibroblastic phenotype in MCT cells, as shown by confocal microscopy and Western blot (Figure 8, A and B). It has been suggested that Src kinases have a critical role in MAPK activation to induce tubuloepithelial cell dedifferentiation and migration—key events during EMT—after cell injury.[19,36] Thus, the possibility that Src kinases could mediate EMT induction by PTHrP(1-36) through ERK1/2 phosphorylation was examined. We observed that a Src kinase inhibitor abolished ILK and E-cadherin alterations (Figure 5C) and ERK1/2 phosphorylation (Figure 7B) triggered by PTHrP(1-36) in MCT cells.

We also aimed to evaluate whether this mechanism of EMT induction by PTHrP(1-36) in MCT cells might occur in human tubuloepithelial cells. We found that PTHrP(1-36) dose-dependently increased the protein expression of the mesenchymal marker vimentin and decreased that of the epithelial marker cytokeratin in HK-2 cells (Figure 9, A and B). Furthermore, these changes induced by this PTHrP peptide were abrogated by the inhibitors tyrphostin AG1478 and U0126, as shown by indirect immunofluorescence (Figure 9A)

EGF seems to cooperate with TGF-β to induce at least some EMT changes in several renal tubuloepithelial cell preparations.[8,14,15] We found here that TGF-β1 and EGF, alone or in combination, were similarly efficient in inducing a myofibro-

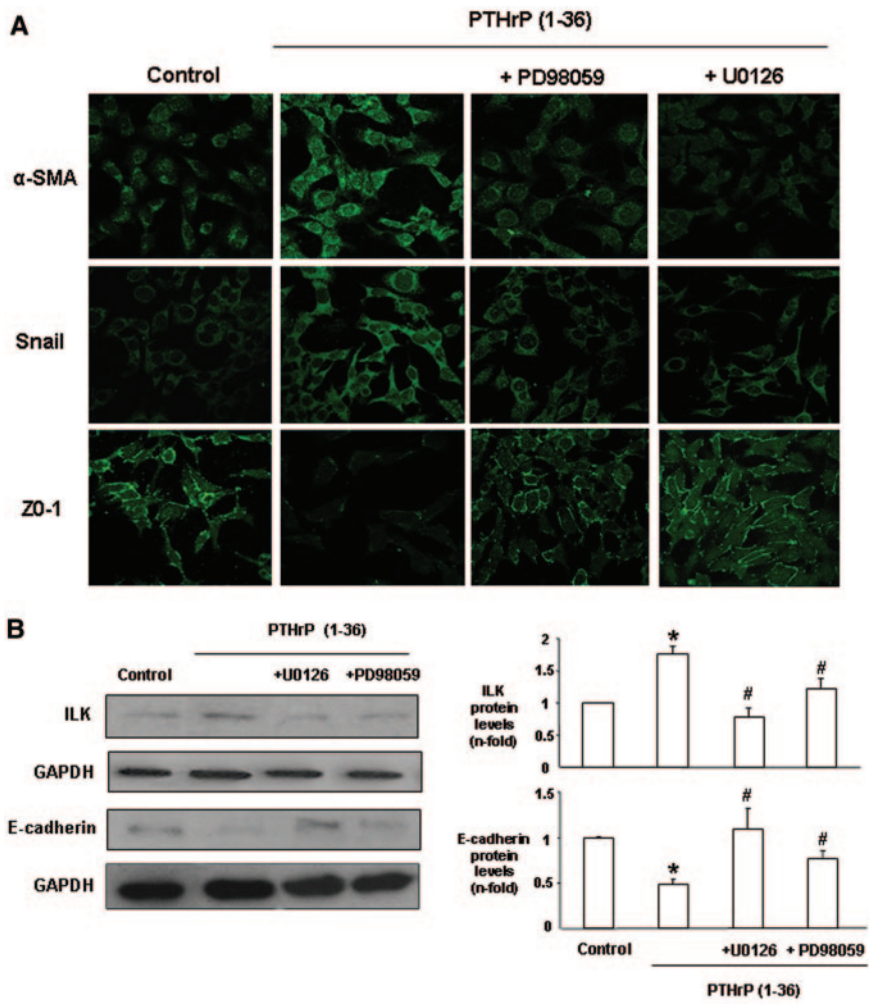

Figure 8. Role of ERK 1/2 activation on PTHrP(1-36)-induced EMT in MCT cells. Cells were preincubated for 1 h with the ERK1/2 inhibitors U0126 and PD98059 (10 μM) and treated with PTHrP(1-36) (100 nM) for 48 h. (A) Confocal microscopy analysis of α-SMA, snail, and ZO-1 immunofluorescence, using specific primary antibodies and a FITC-labeled secondary IgG. This represents the results of three independent observations. (B) Protein levels of ILK and E-cadherin were analyzed by Western blot. Representative autoradiograms are shown. Results are mean ± SEM from at least five independent experiments. *$P < 0.05$ *versus* control; #$P < 0.05$ *versus* PTHrP(1-36) alone.

blast phenotype (Figure 10A) and modulating several EMT-related proteins in MCT cells (Figures 10B and 11); this induction was blocked by an ERK1/2 activation inhibitor (Figures 10, A and B, and 11). In addition, both TGF-β1 and EGF elicited a rapid ERK1/2 phosphorylation that was maintained up to at least 4 h in these cells (Figure 10C). Our recent report suggested that the VEGF system might act as an important mediator of PTHrP(1-36) to induce several profibrogenic actions, including some EMT-related changes, in the obstructed mouse kidney.[24] We found here that U0126 also inhibited the changes elicited by $VEGF_{164}$ in α-SMA and ZO-1, as shown by confocal microscopy, in MCT cells (Figure 11). Of interest, inhibiting either EGFR or ERK1/2 activation abrogated the induction of both TGF-β1 and VEGF mRNA by PTHrP(1-36) in these cells (Figure 2, A and C). Collectively, these data support the hypothesis that ERK1/2 activation is a key pathway in the mechanism underlying EMT induction by any of these factors in renal tubuloepithelial cells.

DISCUSSION

This study further extends our previous studies[24] and shows that PTHrP(1-36) induces a variety of phenotypic changes related to EMT in tubuloepithelial cells. Hence, this peptide induced snail overexpression and its nuclear translocation, associated with the loss of ZO-1 and E-cadherin, key proteins in the maintenance of basolateral polarity and intercellular junctions in the renal tubuloepithelium.[3,5,6] PTHrP(1-36) also induced the phenotypic conversion to a fibroblast-like morphology, related to α-SMA and ILK upregulation. In a previous report, we showed that the VEGF system seemed to have an important role at least in some of these alterations induced by PTHrP(1-36) in tubuloepithelial cells.[24] These results show for the first time that PTHrP(1-36) can also interact with EGFR activation and TGF-β to elicit EMT in these cells. In addition, our *in vivo* data in mice suggest that such interaction might also occur *in vivo* in the damaged kidney.

Current information regarding the interaction between TGF-β and PTHrP in the kidney is scarce. TGF-β has been shown to downregulate the PTHR1 in renal tubuloepithelial cells,[37] but there are no available data about a putative effect of PTHrP on TGF-β in these cells. In this study, we showed that PTHrP(1-36) can increase TGF-β, at both gene and protein secretion levels, in MCT cells. TGF-β blockade by different maneuvers, including a neutralizing antibody, antisense oligonucleotides, decorin, and Smad7 overexpression, was found to diminish renal fibrosis in both experimental models of renal damage and cultured renal cells.[38] TGF-β is a key modulator of the entire EMT process. In tubuloepithelial cells, antagonizing TGF-β diminishes EMT caused by either angiotensin II or high glucose (two pro-fibrogenic factors in the kidney).[33,38] We show here that different strategies for blocking TGF-β, such as a neutralizing antibody, a TRI-ki, and the antagonist decorin, significantly diminished EMT induction by PTHrP(1-36) in MCT cells, strongly suggesting that TGF-β is a downstream mediator of this PTHrP(1-36) action.

In this study, we also show that PTHrP(1-36) can induce EGFR phosphorylation with a pattern of response similar to

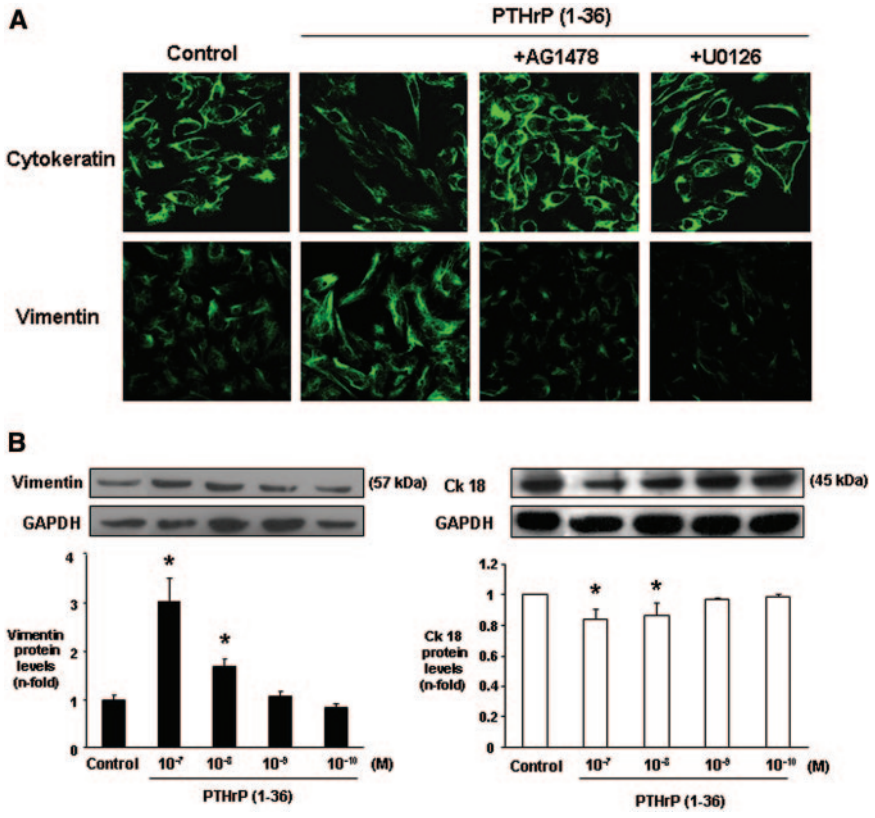

Figure 9. PTHrP(1-36) can promote EMT by interaction with EGFR and ERK1/2 in HK-2 cells. Cells were preincubated for 1 h with or without an ERK1/2 activation inhibitor (U0126, 10 μM) or tyrphostin AG1478 (100 nM) and then were stimulated with or without (control) PTHrP(1-36), at 100 nM (A) or at different concentrations (B), for 48 h in culture medium. (A) Confocal microscopy analysis of cytokeratin and vimentin immunofluorescence was performed using specific primary antibodies and a FITC-labeled secondary IgG. This represents the results of three independent observations. (B) Protein levels of cytokeratin-18 (Ck18) and vimentin were analyzed by Western blot. Representative autoradiograms are shown. Results are mean ± SEM from three independent experiments. *$P < 0.05$ *versus* control.

that elicited by ligands signaling through various GPCRs, such as the calcium receptor in prostate cancer cells, the angiotensin II receptor in vascular smooth muscle cells, and the PTH1R in murine osteoblasts and human embryonic kidney cells.[29,30,39,40] Two main different EGFR transactivation mechanisms induced by GPCRs have been described thus far.[41,42] One of them involves the proteolytic processing of EGFR ligands, which are synthesized as membrane-anchored precursors [*e.g.,* common proheparin-binding (proHB)-EGF], by GPCR-activated MMPs to release soluble EGFR ligands.[39,40,43] Such a mechanism has been described for EGFR transactivation through the PTH1R in HEK-293 cells, which seems to involve PKC activation as a consequence of Gq signaling but not PKA signaling.[30] Alternatively, EGFR can be transactivated by the soluble kinase Src that directly phosphorylates and activates EGFR.[44] These results suggest that the first mechanism seems to account mainly for EGFR transactivation by PTHrP(1-36) in tubuloepithelial cells. Although the true EGFR ligand involved in PTHrP(1-36)-induced EGFR transactivation in these cells is presently unknown, HB-EGF type of ligands are expressed by proximal tubule cells.[44,45] In addition, as occurs in rabbit proximal tubule cells under stress,[19] the ERK1/2 pathway does not seem to be a cause but a consequence of EGFR activation by PTHrP(1-36) in MCT cells. In addition, these findings indicate that EGFR activation by this PTHrP peptide can promote EMT in tubuloepithelial cells. Also consistent with this notion, PKC activation but not PKA signaling seems to be involved in this action of PTHrP(1-36). Our data suggest that Src activation also contributes to PTHrP(1-36)-induced EMT features in MCT cells. In this regard, the role of Src as a regulating kinase during the EMT process in renal tubuloepithelial cells has recently been reported.[19] Moreover, in renal tubuloepithelial MDCK cells, Src activation induces ERK1/2 phosphorylation to promote cell migration.[36] In addition, accumulated evidence suggests a bidirectional association between Src and EGFR, leading to activation of both kinases, which is a mechanism required for many cellular functions including cell migration.[46] Our present results suggest that a similar mechanism might be functional in MCT cells after stimulation with PTHrP(1-36).

Our data also show that ERK1/2 pathway is essential for PTHrP(1-36) to induce EMT changes through EGFR activation and also by modulating TGF-β_1 and VEGF expression in tubuloepithelial cells. Of interest in this regard, both EGF and TGF-β_1 were shown to increase VEGF expression in several tubuloepithelial cell lines, an effect that, at least in the case of TGF-β_1, occurs through ERK1/2 activation.[47,48] Thus, such a mechanism might contribute to the VEGF upregulation by PTHrP(1-36) in MCT cells. In any event, these findings are consistent with an important role of ERK1/2 signaling pathway in the EMT process.[17,49] The latter pathway may activate snail and block transcription of E-cadherin, clauidin, and ocludin, leading to disruption of intercellular junctions in tubuloepithelial cells, which is the first step of EMT. Moreover, E-cadherin repression increases cytoplasmic β-catenin, a key factor in EMT induction.[5,12,32]

These findings further expand those recently reported[24] and strongly support that PTHrP(1-36) can be considered as a new EMT modulator in the kidney. Our data show for the first time that, besides VEGF (as shown in our previous work), EGFR and TGF-β can also be important mediators of this process induced by PTHrP(1-36) in the renal tubular epithelium.

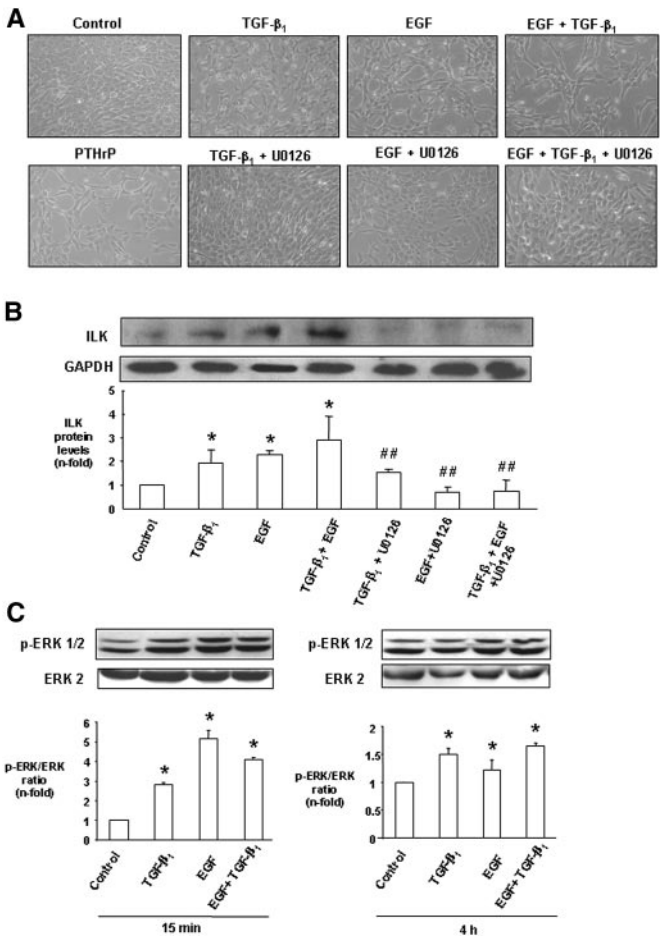

Figure 10. Role of ERK pathway in EMT-related changes by TGF-β_1 and EGF in MCT cells. Cells were preincubated for 1 h with or without an ERK1/2 activation inhibitor (U0126, 10 μM) and then treated with TGF-β_1 (1 ng/ml) and EGF (20 ng/ml), alone or in combination for 48 h. (A) Representative phase-contrast images of MCT cells treated with these factors or not (control) are shown (original magnification, ×200). (B) Changes in ILK protein levels analyzed by Western blot. (C) Time course of ERK1/2 phosphorylation after stimulation with TGF-β and EGF. Representative Western blots are shown. Results are mean ± SEM of three independent experiments. *$P < 0.05$ *versus* control; ##$P < 0.05$ *versus* corresponding factor without U0126.

Moreover, ERK activation seems to be a key downstream event whereby these growth factors might cooperate to induce EMT (Figure 12).

CONCISE METHODS

Cell Cultures

Renal tubuloepithelial MCT cells and the human proximal tubule cell line HK-2, which respond to PTHrP(1-36),[22–24,50] were grown in RPMI 1640 with 10% FBS, supplemented with 1% insulin-transferrin-sodium selenite media (Sigma-Aldrich, St. Louis, MO) and hydrocortisone (36 ng/ml) (HK-2 cells), and antibiotics in 5% CO_2 at 37°C. Subconfluent cells (60,000 cells/cm^2) were incubated with PTHrP(1-36) (100 nM), human recombinant TGF-β_1 (1 ng/ml) (Peprotech, Rocky Hill, NJ), and EGF (20 ng/ml) (Sigma-Aldrich), or mouse recombinant $VEGF_{164}$ (20 ng/ml) (R&D Systems, Minneapolis, MN), in this medium without (HK-2) or with 1% FBS (MCT) for different times. In some experiments, the following inhibitors were added 1 h before PTHrP(1-36): PD98059 (Stressgen Bioreagents, Victoria, BC, Canada); U0126 (Promega, Madison, WI) (each at 10 μM); 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP1) (1 μM); calphostin C (250 nM); (2*R*)-2-[(4-biphenyllylsulfonyl)amino]-3-phenylpropionic acid (MMP-2/MMP-9 inhibitor I] (1 μM); GM6001, a pan-specific inhibitor of MMPs (1 μM) and TRI-ki (10 μM) (Calbiochem; San Diego, CA); Rp-cAMPS (1μM) and decorin (100 nM) (Sigma-Aldrich); and tyrphostin AG 1478 (100 nM) (Alomone Labs, Jerusalem, Israel), an anti-TGF-β–neutralizing antibody that recognizes bovine, mouse, and human TGF-β_1 and β_2 isoforms (1 μg/ml) (R&D, Minneapolis, MN).

Western Blot Analysis

After stimulations, cell extracts in lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X.-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulphonylfluoride, 0.8 μM aprotinin, and a phosphatase-inhibitor cocktail (Set II; Calbiochem)] were obtained for protein analysis. Bradford's method (Pierce, Rockford, IL) was used with BSA as standard. The efficacy of protein loading and transfer to membranes was assessed by α-tubulin and GAPDH.

To analyze changes in EGFR phosphorylation in MCT cells, cell protein extracts (200 μg) were incubated with 0.2 μg rabbit IgG for 1 h at 4°C with shaking. Then, 30 μl of protein A-agarose were added to cell extracts (0.5 ml agarose/2 ml PBS) and incubated for 2 h at 4°C with shaking. After centrifugation for 5 min at 1500 × *g*, supernatants were incubated overnight with a rabbit polyclonal anti-EGFR antibody (Calbiochem; 1:200) at 4 °C with shaking. Protein A-agarose was added, and protein samples were subsequently incubated and centrifuged as described above.

MCT cell protein extracts or the reconstituted immunoprecipitated pellets (for EGFR analysis) (30 to 80 μg/lane) and mouse kidney protein extracts (150 μg/lane), obtained as recently described,[24] were separated on 5 to 10% polyacrylamide-SDS gels under reducing conditions. Samples were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), blocked with 5% defatted milk in 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl with 0.05% Tween-20, and incubated overnight at 4 °C with the following antibodies [dilution, -fold]: mouse monoclonal E-cadherin [2000] (BD Transduction Laboratories, San José, CA); p-tyrosine (PY20) [500] and EGFR [250] (Santa Cruz Biotechnology, Santa Cruz, CA); vimentin [10,000] (BD Pharmigen, Franklin Lakes, NJ); and pan-cytokeratin [1000] (Sigma-Aldrich) antibodies; and rabbit polyclonal ILK [1000] (Santa Cruz Biotechnology); ERK 1/2, or phosphorylated (p)-ERK 1/2 (Thr^{202}/Tyr^{204}) [2000] (Cell Signaling Technology; Beverly, MA); and p-EGFR (p-Tyr^{1068}) [250] (Calbiochem) antibodies. Membranes were subsequently incubated with peroxidase-conjugated IgG and developed by ECL chemiluminescence (GE Healthcare, Buckinghamshire, UK). Densitometric values of fluorogram bands were normalized to those of corresponding α-tubulin or glyceraldehyde 3-phosphate dehydrogenase.

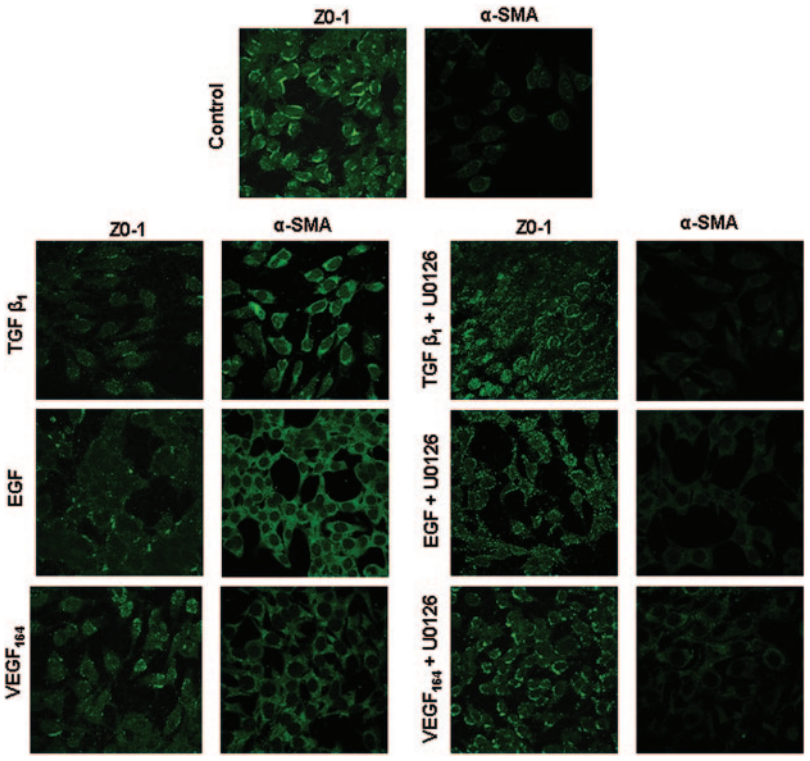

Figure 11. Inhibition of ERK activation abrogated the induction of α-SMA and ZO-1 by TGF-β_1, EGF, or $VEGF_{164}$ in MCT cells. Cells were preincubated for 1 h with or without an ERK1/2 activation inhibitor (U0126, 10 μM) and then treated or untreated (control) with TGF-β_1 (1 ng/ml), EGF (20 ng/ml), or VEGF164 (20 ng/ml) for 48 h. Confocal microscopy analysis of α-SMA and ZO-1 immunofluorescence was performed using specific primary antibodies and a FITC-labeled secondary IgG. This represents the results of three independent observations.

Immunofluorescence

Cells grown on coverslips were stimulated with the agonists, fixed in Merckofix (Merck, Whitehouse Station, NJ), and permeabilized with 0.1% Triton-X100 for 2 min. After blocking with 10% BSA and 10% FBS for 1 h, they were incubated with several primary antibodies [dilution, -fold]: rabbit polyclonal anti-Z0–1[200] (Zymed Laboratories. San Francisco, CA); anti-α-SMA[200] (ABCam, Cambridge, MA);[200] and anti-snail (Sta. Cruz Biotechnology)[200] antibodies; or mouse monoclonal anti-vimentin[200] (BD Pharmigen) and anti-pancytokeratin[200] (Sigma-Aldrich) antibodies for 1 h, followed by a FITC-conjugated secondary antibody [200] or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma-Aldrich) [1000] for 1 h. Absence of primary antibody was used as negative control. Samples were mounted in Mowiol 40-88 (Sigma-Aldrich) and examined using a Leica DM-IRB confocal microscope.

Analysis of mRNA Expression

Total RNA was isolated from MCT cells and mouse kidney samples with Trizol (Invitrogen, Groningen, The Netherlands). cDNA was synthesized using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using 2 μg of total RNA primed with random hexamer primers, following the manufacturer's instructions. Real-time PCR was performed using mouse fluorogenic TaqMan MGB probes and primers designed by Assay-on-Demand gene expression products (Applied Biosystems): TGF-β_1 (Mm001178819_m1), α-SMA (Mm01546133_m1), snail (Mm00441533_g1), and VEGF (Mm00437304_m1). Data were normalized to 18S eukaryotic ribosomal RNA. The mRNA copy numbers were calculated for each sample by the instrument software using Ct value ("arithmetic fit point analysis for the lightcycler"). Results were expressed in copy numbers, calculated relative to unstimulated cells after normalization against 18S.

TGF β_1 Protein Assay

TGF-β_1 protein was measured in the MCT cell-conditioned medium after treatment with PTHrP(1-36) (100 nM) for 48 h, using a commercial ELISA (BD Sciences, San Diego, CA) following the manufacturer's instructions. Active TGF-β_1 was determined in 100 μl of the cell-conditioned medium (stored at -80°C). Inactive TGF-β_1 was converted to the active form by incubating these cell culture supernatants with 10 N HCl for 10 min, followed by neutralization with 10 N NaOH/0.5 M HEPES. Protein content was determined by the BCA method (Pierce). TGF-β_1 activity was quantified by comparison with a standard curve of human TGF-β_1.

Mouse Model of Unilateral Ureteral Obstruction

Transgenic mice with targeted overexpression of PTHrP to the renal proximal tubule (PTHrP-TG) and their control littermates were used.[22–24] Unilateral ureteral obstruction (UUO) was performed under anesthesia by ligating the left ureter of each animal with 3-0 silk—at two locations and cutting between the ligatures—through an abdominal incision, as previously reported.[23,24] Four days thereafter, mice were killed, and the obstructed kidneys were collected. Sham-operated mice, which had their ureters manipulated but not ligated, served as controls. Kidney portions were separated from each mouse and stored at -80°C for subsequent analysis. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Fundación Jiménez Díaz.

Statistical Analysis

Results throughout the text are expressed as mean ± SEM. Differences between agonist-treated groups and controls were assessed by one-way ANOVA, followed by the *post hoc* Bonferroni or Dunnett test, or Mann-Whitney test, as appropriate. $P < 0.05$ was considered significant. Statistical analysis was conducted using the SPSS statistical software (version 11.0; SPSS, Chicago, IL).

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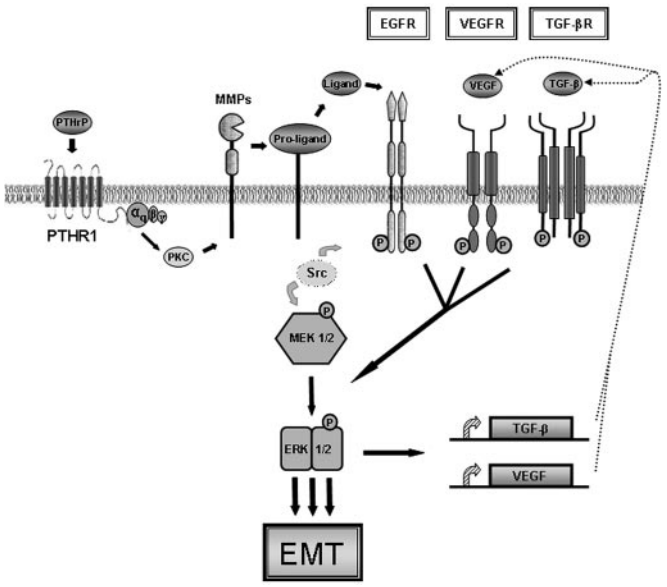

Figure 12. Proposed mechanism for ERK1/2 activation as a key signaling pathway targeted by PTHrP(1-36) through interaction with EGFR, TGF-β, and VEGF in renal tubuloepithelial cells.

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DISCLOSURES

None.

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4. La transactivación del EGFR regula la inflamación renal experimental inducida por TWEAK.

TWEAK se une a su receptor Fn14 desencadenando diversas respuestas biológicas, incluidas proliferación celular, apoptosis, angiogénesis e inflamación (Locksley et al., 2001). El Factor de Necrosis Tumoral (TNF-α) y TWEAK parecen ser citoquinas no redundantes en la lesión renal ya que aunque comparten ciertas respuestas biológicas dependientes de NF-κB, difieren en otras. Se ha descrito que TNF-α induce la transactivación del EGFR en células epiteliales gastrointestinales (Hobbs et al., 2013), pero se desconoce el efecto de TWEAK sobre esta ruta de señalización. Estudios experimentales han demostrado que la transactivación del EGFR esta implicada en la fibrosis renal (Lautrette et al., 2005), pero apenas existen datos acerca de su papel en la respuesta inflamatoria. La transactivación de EGFR está mediada por MMPs de la familia ADAMs, que actúan liberando los ligandos inactivos unidos a membrana. En riñón se ha descrito que ADAM17 y los ligandos HB-EGF y TGF-α son los más relevantes en este proceso (Ueno et al., 2005, Doedens et al., 2003, Lautrette et al., 2005, Prewett et al., 1998). En este trabajo experimental se aborda el objetivo 3 de la tesis que ha consistido en investigar si TWEAK es capaz de regular la vía de señalización del EGFR en riñón, así como su potencial implicación en las respuestas renales inducidas por esta citoquina.

El efecto *in vivo* de TWEAK se estudió mediante la administración sistémica de la forma soluble de la citoquina recombinante TWEAK en ratón. Al cabo de 24 horas, TWEAK aumentó la fosforilación del EGFR en riñón, localizada principalmente en células tubuloepiteliales. El bloqueo de la activación de esta ruta, mediante un inhibidor de la quinasa del EGFR (Erlotinib), inhibió la respuesta inflamatoria inducida por TWEAK en riñón, disminuyendo el número de células inflamatorias en el intersticio renal y la inducción de factores proinflamatorios. En células tubuloepiteliales en cultivo, mediante técnicas de del silenciamiento génico y/o inhibición farmacológica, se observó que TWEAK se une a su receptor Fn14 e induce la transactivación del EGFR por un mecanismo regulado por la activación de ADAM17 y la liberación de los ligandos HB-EGF y TGF-α. Esta transactivación del EGFR desencadena diversas rutas de señalización, incluidas la activación de la quinasa ERK, que conducen a la regulación de factores proinflamatorios. La estimulación *in vitro* con los ligandos recombinantes TGF-α y HB-EGF también aumentó la expresión génica de mediadores proinflamatorios. Estos datos muestran que la ruta del EGFR regula la respuesta inflamatoria renal. Para determinar la implicación de ADAM17 en las acciones renales de TWEAK *in vivo* se utilizó un inhibidor farmacológico especifico de ADAM17 (WTACE-2). El bloqueo de ADAM17 previno la respuesta inflamatoria inducida por TWEAK. En último lugar, se estudiaron posibles vías de señalización reguladas por la ruta TWEAK/ADAM17/EGFR, con especial atención a NF-κB, implicado en la respuesta inflamatoria inducida por TWEAK en riñón. Los estudios *in vivo* e *in vitro* realizados demuestran que la transactivación del EGFR por TWEAK es independiente de la activación la vía canónica de NF-κB.

Todos estos datos sugieren que la modulación de la vía ADAM-17/EGFR podría ser una nueva opción terapéutica en patologías renales asociadas a inflamación.

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ORIGINAL PAPER

TWEAK transactivation of the epidermal growth factor receptor mediates renal inflammation

Sandra Rayego-Mateos,[1] Jose Luis Morgado-Pascual,[1] Ana Belen Sanz,[2] Adrian Mario Ramos,[2] Satoru Eguchi,[3] Daniel Batlle,[4] Janos Pato,[6] Gyorgy Keri,[5,6] Jesus Egido,[2] Alberto Ortiz[2] and Marta Ruiz-Ortega[1,2]*

[1] *Cellular Biology in Renal Diseases Laboratory, Universidad Autónoma, Madrid, Spain*
[2] *Division of Nephrology and Hypertension, IIS-Fundación Jiménez Díaz-UAM/IRSIN, Madrid, Spain*
[3] *Temple University School of Medicine, Philadelphia, PA, USA*
[4] *Northwestern University, Feinberg School of Medicine, Chicago, IL, USA*
[5] *Pathobiochemistry Research Group of the Hungarian Academy of Science at Semmelweis University, Budapest, Hungary*
[6] *Vichem Chemie Ltd, Budapest, Hungary*

**Correspondence to: M Ruiz-Ortega, Cellular Biology in Renal Diseases Laboratory, Fundación Jiménez Díaz, Avda. Reyes Católicos 2, 28040 Madrid, Spain. E-mail: mruizo@fjd.es*

Abstract

TWEAK, a member of the TNF superfamily, binds to the Fn14 receptor, eliciting biological responses. EGFR signalling is involved in experimental renal injury. Our aim was to investigate the relationship between TWEAK and EGFR in the kidney. Systemic TWEAK administration into C57BL/6 mice increased renal EGFR phosphorylation, mainly in tubular epithelial cells. *In vitro*, in these cells TWEAK phosphorylated EGFR via Fn14 binding, ADAM17 activation and subsequent release of the EGFR ligands HB-EGF and TGFα. *In vivo* the EGFR kinase inhibitor Erlotinib inhibited TWEAK-induced renal EGFR activation and downstream signalling, including ERK activation, up-regulation of proinflammatory factors and inflammatory cell infiltration. Moreover, the ADAM17 inhibitor WTACE-2 also prevented those TWEAK-induced renal effects. *In vitro* TWEAK induction of proinflammatory factors was prevented by EGFR, ERK or ADAM17 inhibition. In contrast, EGFR transactivation did not modify TWEAK-mediated NF-κB activation. Our data suggest that TWEAK transactivates EGFR in the kidney, leading to modulation of downstream effects, including ERK activation and inflammation, and suggest that inhibition of EGFR signalling could be a novel therapeutic tool for renal inflammation.

Keywords: TWEAK; EGFR; ADAM17; inflammation; renal

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No conflicts of interest were declared.

Introduction

Tumour necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily, involved in multiple biological processes, including the immune response and tissue repair [1–3]. TWEAK is synthesized as a type II transmembrane protein, after enzymatic processing by furin results in a biologically active soluble cytokine [4]. There is only one receptor that binds soluble TWEAK with physiological affinity, fibroblast growth factor-inducible 14 (Fn14) [4–6]. Fn14 is a type I transmembrane protein of the TNF receptor superfamily that lacks a cytoplasmic death domain, and therefore TWEAK actions do not mimic those of TNF. Fn14 engagement by TWEAK recruits TNFR-associated factor (TRAF) adapter molecules and activates many intracellular signalling systems, such as the mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) [7,8]. TWEAK/Fn14 activation regulates several cellular responses, including proliferation, angiogenesis, induction of inflammatory cytokines and, under some experimental conditions, apoptosis [2,9,10]. TWEAK and Fn14 are up-regulated in several experimental and human renal diseases [9–11]. TWEAK/Fn14 blockade, using genetically modified mice or neutralizing antibodies, ameliorated experimental renal diseases, including acute renal injury, lupus and nephrotoxic nephritis [12–17]. TWEAK/Fn14 targeting is undergoing clinical trials and an improved understanding of TWEAK actions may guide future development of new therapeutic approaches [9].

Blockade of the epidermal growth factor receptor (EGFR) by genetic or pharmacological approaches ameliorates experimental renal disease progression, mainly by diminishing fibrosis [18,19]. EGFR can be activated by direct binding of specific ligands present in the cell environment, or it can be 'transactivated' by extracellular stimuli, such as agonists of G protein-coupled receptors, cytokines (including

TNFα), integrins, ion channels and other physical stimuli [20–24], but there are no data on TWEAK. EGFR transactivation is regulated by specific proteases of the family of membrane-anchored disintegrin-metalloproteases (ADAMs), of which ADAM17 (also named TACE) is the best-characterized member [24,25]. ADAM17 is involved in EGFR transactivation in renal fibrosis [26]. EGFR ligands exist as inactive pro-forms anchored to the extracellular membrane; however, ADAMs' proteolytic post-translational 'ectodomain shedding' of EGFR ligands leads to the release of the extracellular domain from the membrane-anchored form to generate bioactive soluble factors, which bind to and activate EGFR [25,27]. There are seven EGFR ligands: EGF; heparin binding EGF-like growth factor (HB-EGF); transforming growth factor-α (TGFα); amphiregulin; β-cellulin; epigen; and epiregulin [23,28,29]. Some of them are processed by ADAM17 [23,29] and are of potential interest in renal diseases [19]. Our aim was to investigate whether TWEAK could transactivate EGFR in the kidney, the molecular mechanism involved and the contribution of this molecular pathway to TWEAK-induced renal responses.

Methods

Cultured cells

Human (HK2; ATCC CRL-2190 cell line) and murine (MCT, kindly donated by Dr Eric Neilson, Vanderbilt University) kidney proximal tubule epithelial cells were grown as described [30].

Reagents

Recombinant proteins: human soluble TWEAK (Millipore), HB-EGF and TGFα (Peprotech). The inhibitors used were Erlotinib (EGFR kinase, Vichem), TAPI-2 (ADAM17; Enzo Life Sciences), CRM197 (HB-EGF, Sigma), U0126 and PD98059 (ERK; Calbiochem) and TGFα-neutralizing antibody (AbCam). DMSO, used as a solvent in some cases, had no effect on cell viability or on gene expression levels (not shown).

Design of the experimental model

All animal procedures were performed with prior approval by the Ethics Committee of Health of the IIS-FJD, following the guidelines for animal research in the European Community. Studies were done in female C57BL/6 mice, aged 9–12 weeks, weight 20 g, obtained from and maintained in IIS animal facilities under special pathogen-free conditions.

The mice received an intraperitoneal (ip) injection of 0.5 µg TWEAK/mouse (dissolved in saline) and sacrificed 24 h later. Controls were injected with saline ($n = 8–10$ mice/group). TWEAK endotoxin level were < 0.1 ng/mg, confirmed by MALDI–TOF (not shown). The treatments were started 24 h prior to TWEAK administration. The mice were treated daily with the EGFR kinase inhibitor Erlotinib (40 mg/kg/day ip) or the ADAM17 inhibitor WTACE-2 (100 mg/kg/day ip; Pfizer). They were sacrificed under anaesthesia (ketamine and xylazine), perfused with cold saline and the kidneys were extracted. Then portions were fixed in formalin buffer for immunohistochemistry studies or immediately frozen in liquid nitrogen for gene and protein studies.

Renal histology and immunohistochemistry

Paraffin-embedded kidney sections (3 µm) were stained using conventional methods. Antigen retrieval was performed using the PTlink system (Dako Diagnostics) with sodium citrate buffer (10 mM) adjusted to pH 6–9, depending on the immunohistochemical marker, followed by immunohistochemical staining in a Dako Autostainer. The steps were: (a) endogenous peroxidase blockade; (b) primary antibodies incubation—anti-CD3 (1:300; Dako) or anti-F4/80 (1:5000; Serotec) or anti-pEGFR1173 (1:200; Cell Signalling); (c) washing; and (d) Duoflex Doublestain EnVision™ treatment, using 3,3′-diaminobenzidine as chromogen. For F4/80 staining, a rabbit anti-rat linker was used before EnVision. The sections were counterstained with Carazzi´s haematoxylin. The intensity of the reactive mark was obtained using Image-Pro Plus software. For each sample (processed by duplicate in a blinded manner) the average value was obtained from the analysis of four fields (×20 objective) as density/mm^2 or percentage stained area versus total analysed area. Data are expressed as n-fold increase over control mice. Negative controls include non-specific immunoglobulin and no primary antibody (not shown).

Immunofluorescence was used to discriminate between proximal tubular and distal tubular expression of pEGFR. For these studies, sections were stained with anti-pEGFR (1:200; Dako), secondary AlexaFluor® 633 conjugated goat anti-mouse (1:300; Invitrogen), and also incubated with the proximal tubule marker FITC–fluorescein-conjugated Lotus Tetragonolobus Lectin (1:33; Sigma) or the collecting tubule marker FITC–fluorescein-conjugated Dolichos biflorus agglutinin lectin (1:33; Sigma), as described [31].

Gene silencing

Gene silencing in cultured cells was performed using either predesigned siRNA corresponding to EGFR, ADAM17 or Fn14, or their corresponding scrambled siRNAs (Ambion). Subconfluent cells were transfected for 24 h with 25 nM siRNA, using 50 nM Lipofectamine RNAiMAX (Invitrogen) or treated only with lipofectamine vehicle, according to the manufacturer's instructions. Then the cells were incubated with 10% heat-inactivated fetal bovine serum (FBS) for 24 h, followed by 24 h in serum-free medium before the experiments.

Protein studies

Proteins were obtained from treated cells or mouse kidneys, using lysis buffer [50 mM Tris–HCl, 150 M NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% Octylphenyl-polyethylene glycol (IGEPAL®), 10 µl/ml proteinase inhibitors cocktail, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.2 mM orthovanadate]. To determine protein content, the BCA method was used.

For western blotting, cell (25 µg/lane) and kidney (100–150 µg/lane) protein extracts were separated on 6–12% polyacrylamide–SDS gels under reducing conditions. The samples were then transferred onto nitrocellulose membranes (BioRad), blocked with tris buffered saline (TBS)/5% defatted milk/0.05% Tween-20 and incubated overnight at 4 °C with the following antibodies (dilution): phosphorylated EGFR on tyrosine 1068 (p-EGFR$_{1068}$, (1:250); Calbiochem), or tyrosine 1173 p-EGFR$_{1173}$ (1:250), p-p65 (1:250), Fn14 (1:1000) (all from Cell Signaling), ADAM17 (1:1000; Abcam), EGFR(1:250), ERK 1/2 (1:200) or p65 (1:250) (all from Santa Cruz Biotechnology). The membranes were subsequently incubated with peroxidase-conjugated IgG secondary antibody and developed using an ECL chemiluminescence kit (Amersham). Loading controls were done using antibodies against GAPDH (1:10000; Chemicon), α tubulin (1:5000; Sigma-Aldrich) or histone H1 (1:250; Santa Cruz Biotechnology) for nuclear proteins or total protein levels in phosphorylation studies. Autoradiographs were scanned using a Gel Doc™ EZ imager and analysed using Image Lab 3.0 software (BioRad).

The ELISA method was used to evaluate levels of the chemokines CCL-2 and CCL-5 (eBioscience) and the release of EGFR ligands, TGFα and HB-EGF (RayBiotech). Levels were quantified by comparison with a standard curve. In renal samples, total protein content was determined by the BCA method, and equal amounts were analysed. Data are expressed as *n*-fold increase over the mean of control levels.

Gene expression studies

Total RNA was isolated from cells and mouse kidney samples with Trizol (Invitrogen). The cDNA was synthesized using a High-Capacity cDNA Archive Kit (Applied Biosystems), using 2 µg total RNA primed with random hexamer primers. Multiplex real-time PCR was performed using Applied Biosystems expression assays: CCL-2 Mm00441242_m1; CCL-5 Mm_ 01302428_m1; IL-6 Mm_00446190_m1; IP-10 Mm_00445235_m1; CCL-20 Mm_01268754_m1; ICAM-1 Mm_00516023_m1; Osteopontin Mm_00436767_m1; HB-EGF Mm_00439306_m1; TGFα Mm_00446232_m1. The data were normalized to *18S*; 4210893E (VIC). The mRNA copy numbers were calculated for each sample by the instrument software, using the C_T values. The results are expressed in copy numbers, calculated relative to unstimulated cells or control mice, after normalization against *18S*.

Statistical analysis

All results are expressed as mean ± SEM. Differences between agonist-treated groups and controls were assessed by Mann–Whitney U test; $p < 0.05$ was considered significant. Statistical analysis was conducted using SPSS v. 11.0 statistical software.

Results

TWEAK induces EGFR phosphorylation in the kidney

EGFR activation is characterized by tyrosine autophosphorylation [32,33]. Systemic administration of soluble TWEAK into mice increased renal levels of phosphorylated EGFR compared to control mice (Figure 1A). In the kidney, EGFR is mainly expressed in tubular cells [29]. Immunohistochemistry and immunofluorescence, using antibodies that recognized phosphorylated-EGFR on tyrosines 1173 and 1068, respectively, revealed that TWEAK activated EGFR mainly in proximal tubular cells *in vivo* (Figure 1B–D).

TWEAK induces EGFR transactivation through Fn14 binding in cultured tubular epithelial cells

Next, *in vitro* studies were performed to further investigate TWEAK–EGFR pathway activation. In cultured murine tubular epithelial cells (MCT cells), TWEAK-induced EGFR activation was dose- and time-dependent, starting as early as 5 min and peaking after 15 min, with a maximal response at 100 ng/ml TWEAK (Figure 2A, B). Similar results were observed in human tubular epithelial cells (HK2 cells, not shown). In HK2 cells, EGFR-specific activation was demonstrated by two approaches: pharmacological inhibition and gene silencing. Pre-incubation with the specific EGFR kinase inhibitor Erlotinib blocked TWEAK-induced EGFR activation (Figure 2C). Transfection with an EGFR siRNA resulted in very low EGFR levels (Figure 2D) and no EGFR phosphorylation was observed following treatment with TWEAK. In contrast, increased EGFR phosphorylation was observed in response to TWEAK stimulation in control cells, or in cells transfected with scrambled siRNA or incubated with transfection reagent alone (non siRNA added) (Figure 2D).

Involvement of the Fn14 receptor in TWEAK-induced EGFR activation was studied by a gene-silencing approach. Transfection of HK2 cells with Fn14 siRNA down-regulated Fn14 protein levels compared to control cells, either transfected with scrambled siRNA or with non siRNA (Figure 2E). In Fn14-silenced TWEAK-treated cells, phosphorylated-EGFR levels were significantly lower than in TWEAK-treated controls (either non siRNA or scrambled siRNA) (Figure 2E). These results show that TWEAK engages Fn14 to transactivate EGFR in tubular epithelial cells.

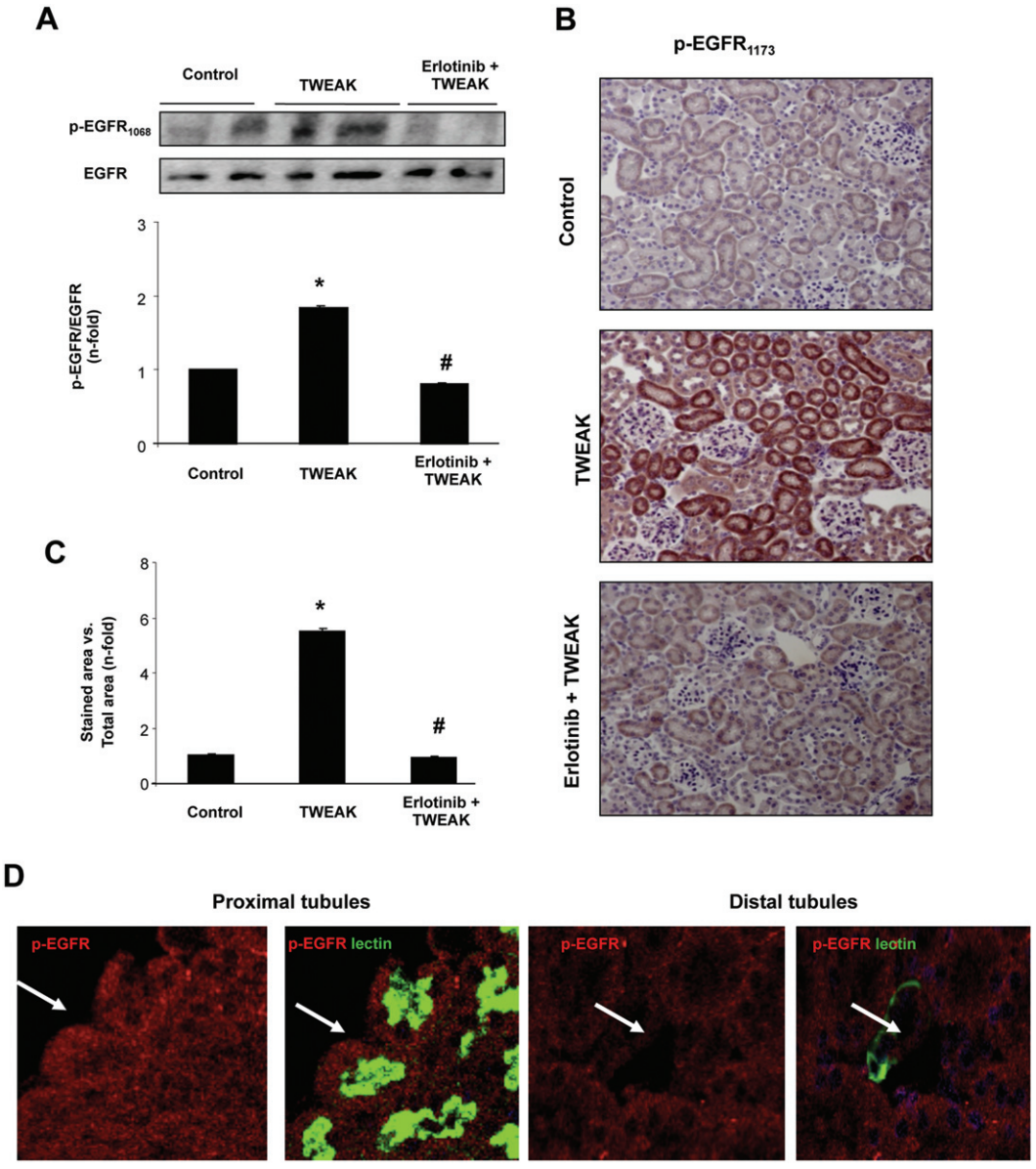

Figure 1. TWEAK induces EGFR phosphorylation in the kidney. C57BL/6 mice were injected (ip) with recombinant TWEAK (0.5 µg/mouse) or vehicle (saline) and sacrificed 24 h later. Some mice were also pretreated 24 h before with Erlotinib (EGFR kinase inhibitor, 40 mg/kg/day). (A) EGFR activation was assessed as EGFR phosphorylation by western blot in total kidney protein extracts, using an antibody against tyrosine 1068-phosphorylated EGFR (p-EGFR$_{1068}$). Total EGFR levels were used as loading control. (B) Immunohistochemistry of active EGFR (using an antibody against EGFR phosphorylated on tyrosine 1173, p-EGFR$_{1173}$) showed positive immunostaining, mainly in tubular epithelial cells. (D) Co-localization of p-EGFR (red) staining with markers of proximal (Lotus Tetragonolobus lectin, green) or distal (Dolichos biflorus agglutinin lectin, green) tubules in TWEAK-injected mice. Proximal tubules are the main sites of p-EGFR staining. Figures show one or two representative mice/group. (A, C) Quantification of the western blot and immunostaining, respectively, expressed as mean ± SEM of 8–10 mice/group: $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus TWEAK

The EGFR kinase inhibitor Erlotinib diminishes TWEAK-induced renal inflammation

To investigate the *in vivo* effect of EGFR blockade in TWEAK-induced renal damage, mice were treated with the EGFR kinase inhibitor Erlotinib, a small molecule tyrosine kinase inhibitor that targets the receptor catalytic domain of EGFR. Erlotinib diminished renal phosphorylated-EGFR levels in TWEAK-injected mice to values similar to those in control mice (Figure 1A–C). TWEAK administration causes an inflammatory response in the kidney [13]. In TWEAK-injected mice, EGFR kinase inhibition significantly diminished the number of infiltrating interstitial monocytes/macrophages (F4/80^{+} cells) and T lymphocytes (CD3^{+} cells) (Figure 3A, B). Erlotinib inhibited TWEAK-induced up-regulation of proinflammatory gene expression, including cytokines (IL-6), chemokines (CCL-2, CCL-5, CCL-20, IP-10 and osteopontin), receptors (CCR2) and adhesion molecules (ICAM-1) (Figure 4A), as found in human cells (not shown). EGFR kinase inhibition also blocked chemokine production, as observed in the case of CCL-2 and CCL-5 (Figure 4B). In cultured murine

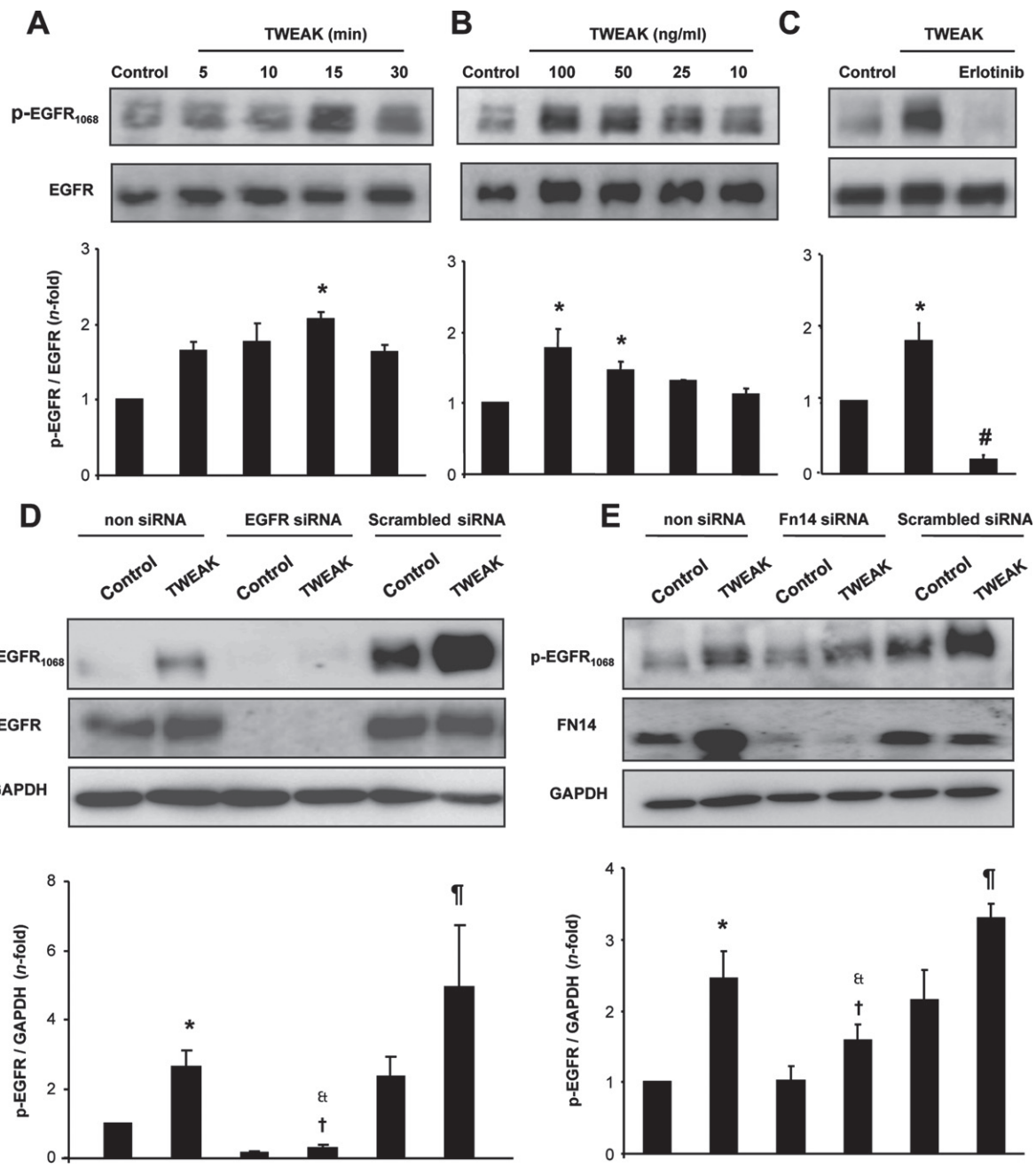

Figure 2. TWEAK via Fn14 induces EGFR phosphorylation in cultured tubular epithelial cells. Murine tubular epithelial cells (MCTs) were treated with 100 ng/ml TWEAK for (A) increasing times or (B) with several concentrations of TWEAK (range 10–100 ng/ml) for 15 min. (C) Human tubular epithelial (HK2) cells were treated with 100 ng/ml TWEAK for 15 min. At some points, cells were pre-incubated for 1 h with the EGFR kinase inhibitor Erlotinib (10 μM). Figures show a representative Western blot experiment and data expressed as mean ± SEM of three independent experiments: $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus TWEAK. Gene silencing of *EGFR* (D) or *Fn14* (E) inhibits TWEAK-induced EGFR phosphorylation in cultured tubular epithelial cells. HK2 cells were transfected with an EGFR siRNA, a Fn14 siRNA or scrambled siRNA or incubated with transfection reagent alone (non-siRNA), as described in Methods. The cells were treated with 100 ng/ml TWEAK for 15 min. EGFR phosphorylation was evaluated by western blot, using an antibody against p-EGFR$_{1068}$. Total *Fn14*, *EGFR* or *GAPDH* levels were used as silencing or loading controls. (D, E) Representative western blot experiment; quantification expressed as the ratio p-EGFR:total EGFR as mean ± SEM of five independent experiments: $^{*}p < 0.05$ versus control (untreated) untransfected cells (non siRNA added). $^{\P}p < 0.05$ versus untreated scramble siRNA-transfected cells. $^{\dagger}p < 0.05$ versus TWEAK-treated scramble siRNA-transfected cells. $^{\&}p < 0.05$ versus TWEAK-treated untransfected cells.

tubular epithelial cells, pre-incubation with Erlotinib also inhibited TWEAK-induced expression of pro-inflammatory genes (Figure 4C). These data show that inhibition of EGFR activation diminishes TWEAK-induced inflammatory responses in the kidney and cultured renal cells.

TWEAK–EGFR pathway activation is linked to downstream ERK activation and induction of pro-inflammatory factors

Upon ligand binding, EGFR can be phosphorylated on different tyrosine residues, leading to ligand-specific

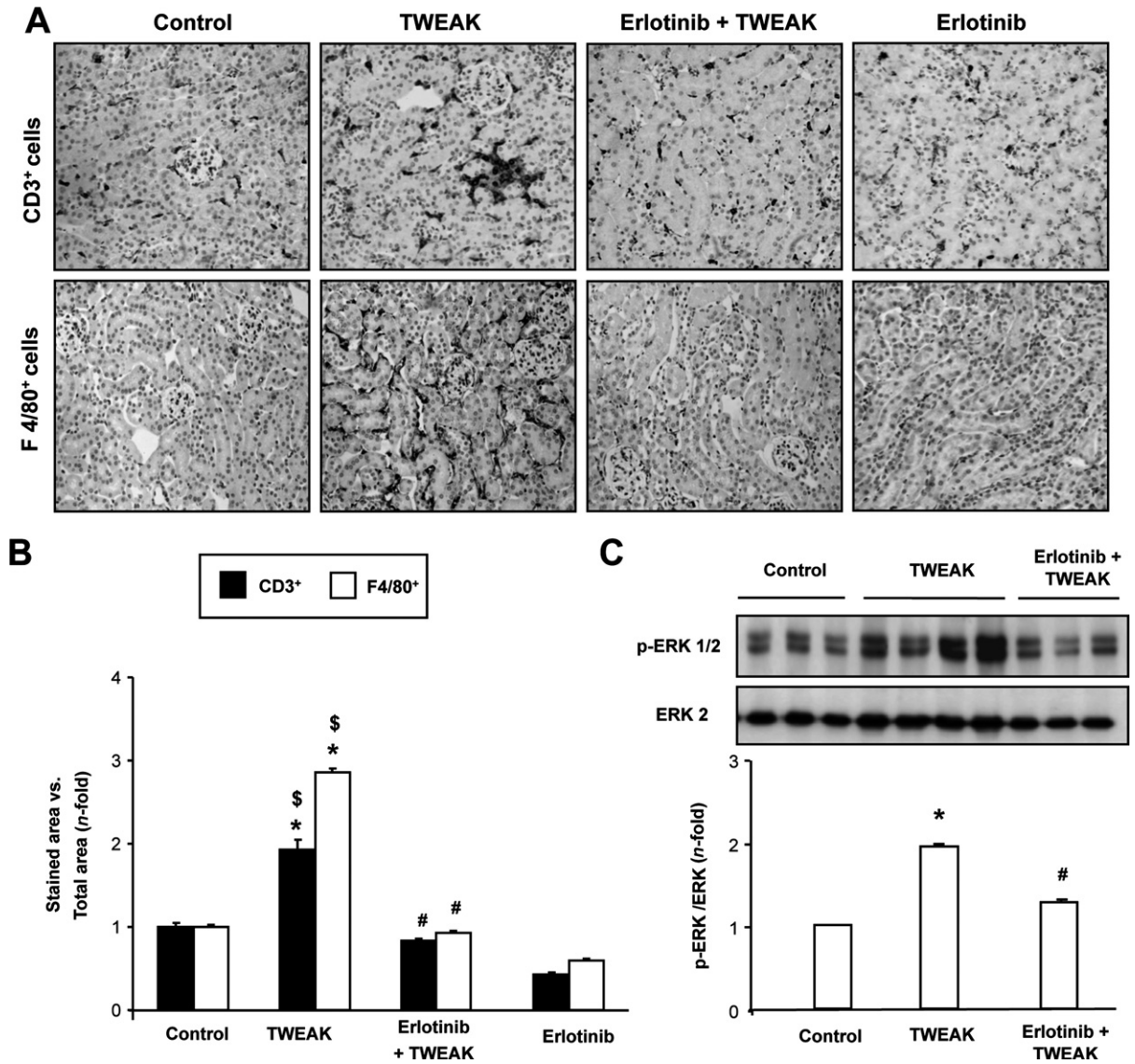

Figure 3. EGFR kinase inhibition ameliorates TWEAK-induced renal injury. Mice were treated with Erlotinib (EGFR kinase inhibitor, 40 mg/kg/day), starting 24 h before TWEAK (0.5 μg/mouse) or saline administration, and sacrificed 24 h later. Erlotinib inhibits TWEAK-induced inflammatory cell infiltration in the kidney but had no effect on saline-injected mice. In paraffin-embedded kidney sections, immunohistochemistry using anti-F4/80 and anti-CD3 antibodies identified monocytes/macrophages and T lymphocytes, respectively. (A) Representative animals from each group; magnification × 200. (B) Staining quantification expressed as mean ± SEM of 8–10 animals/group: $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus TWEAK. (C) Western blot of total kidney protein, using an antibody against phosphorylated ERK1/2 (p-ERK). Figure shows three or four representative mice from each group and, in the lower panel, the quantification, as the ratio pERK:total ERK, as mean ± SEM of 8–10 mice/group. Total ERK levels were used as loading control: $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus TWEAK; $^{\$}p < 0.05$ versus Erlotinib alone

activation of downstream signal transduction pathways. EGFR phosphorylation on tyrosine 1068 and 1173 is involved in ERK signalling [32,33]. TWEAK-injected mice displayed phosphorylation on these tyrosines (Figure 1) and increased ERK1/2 phosphorylation compared to controls (Figure 3C). Both responses were inhibited by EGFR kinase inhibition (Figures 1, 3C). In accordance with our earlier studies, in tubular epithelial cells TWEAK induces rapid ERK1/2 phosphorylation that was diminished by EGFR silencing (Figure 4D). Previous studies showed that TWEAK increases pro-inflammatory factors [13,34], and activates ERK in renal cells [14], but the relationship between these processes has not been demonstrated. ERK inhibition, by U0126 or PD98059, markedly diminished TWEAK-induced pro-inflammatory gene expression (Figure 4C), demonstrating that TWEAK, via ERK, regulates inflammatory related-factors in cultured tubular cells. In summary, these data link EGFR activation by TWEAK to ERK and inflammation.

TWEAK induces EGFR transactivation via ADAM17

The Fn14 receptor lacks tyrosine kinase activity in its cytoplasmic domain, therefore our data suggest that TWEAK binding to Fn14 triggers additional intracellular mechanisms that mediate EGFR transactivation. Among these mechanisms, ADAM17 has special

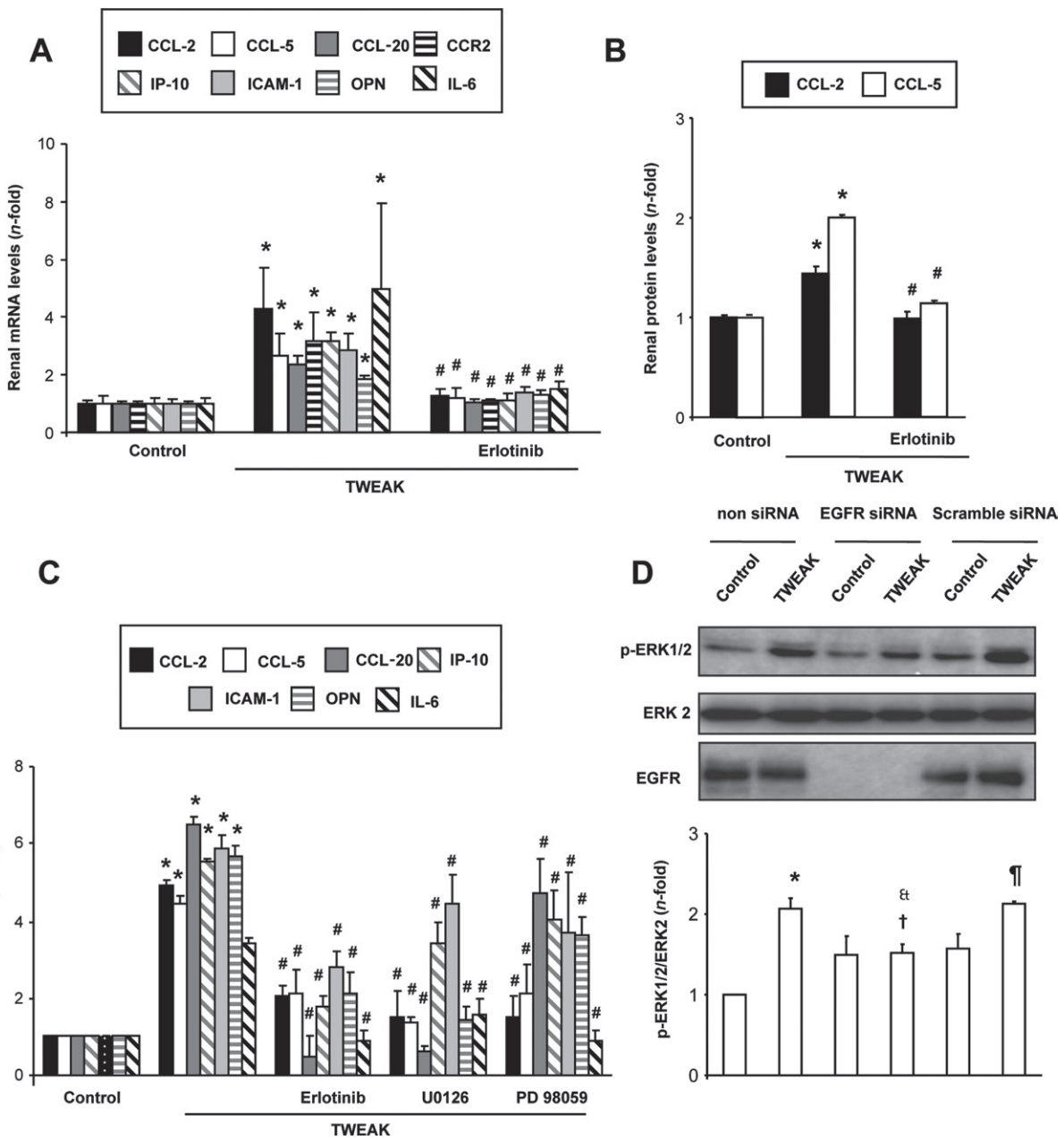

Figure 4. EGFR signalling blockade inhibits TWEAK-mediated pro-inflammatory factors up-regulation in the kidney *in vivo* and *in vitro*. (A) RNA was obtained from total renal extracts, and pro-inflammatory gene expression levels were determined by real-time PCR. (B) Kidney CCL2 and CCL5 protein levels were evaluated by ELISA. (C) Inhibitors of EGFR or ERK decrease TWEAK-induced pro-inflammatory genes over-expression in cultured murine tubular epithelial cells. Cells were pre-incubated for 1 h with 10 μM Erlotinib or the ERK inhibitors U0126 and PD98059 before stimulation with 100 ng/ml TWEAK for 6 h. Data are expressed as mean ± SEM of 8–10 animals/group or six *in vitro* experiments: $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus TWEAK. (D) *EGFR* gene silencing inhibits TWEAK-mediated ERK phosphorylation. HK2 cells were transfected with an EGFR siRNA or scrambled siRNA or incubated with transfection reagent alone (non-siRNA), as described in Methods. Then cells were stimulated or not with 100 ng/ml TWEAK for 15 min. Figure shows a representative western blot of ERK phosphorylation and the lower panel data are mean ± SEM of three independent experiments. Total *EGFR*, *ERK* or *GAPDH* levels were used as loading/silencing controls: $^{*}p < 0.05$ versus control (untreated) untransfected cells (non siRNA added). $^{¶}p < 0.05$ versus untreated scramble siRNA-transfected cells. $^{†}p < 0.05$ versus TWEAK-treated scramble siRNA transfected cells. $^{ξ}p < 0.05$ versus TWEAK-treated untransfected cells.

relevance in the kidney [26], therefore we first evaluated whether TWEAK could regulate ADAM17. In TWEAK-injected mice, renal ADAM17 gene and protein levels were significantly increased compared to control mice (Figure 5A, B). In cultured tubular epithelial cells TWEAK also up-regulated ADAM17 gene expression, as early as at 15 min, peaking at 1 h and remaining elevated after 24 h, decreasing thereafter to control levels (Figure 5C).

Next, ADAM17-mediated EGFR transactivation was investigated. In cultured human tubular cells, ADAM17 blockade, by gene silencing or pharmacological inhibition using TAPI-2, markedly diminished TWEAK-induced EGFR phosphorylation

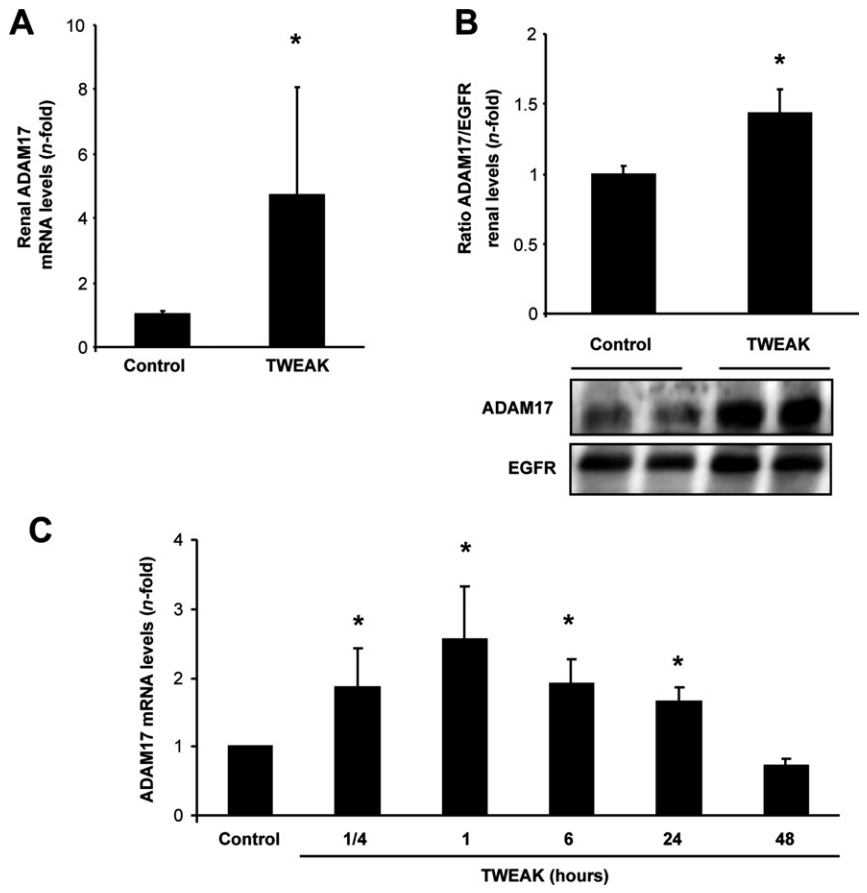

Figure 5. TWEAK regulates ADAM17 in the kidney and in cultured tubular epithelial cells. In total renal extracts from mice injected with TWEAK or saline (control), ADAM17 gene expression was evaluated by real-time PCR (A) and protein levels by western blot (B). EGFR was used as loading control. (C) Murine tubular epithelial cells (MCTs) were treated with 100 ng/ml TWEAK for increasing times. (A, B) Data expressed as mean ± SEM of 8–10 animals/group and in (C) of four *in vitro* experiments: $^*p < 0.05$ versus control

and downstream ERK activation (Figure 6A–C). Moreover, ADAM17 inhibition significantly prevented the up-regulation of pro-inflammatory genes induced by TWEAK (Figure 6D). In TWEAK-injected mice, treatment with the ADAM17 inhibitor WTACE2 diminished phosphorylation levels of EGFR and ERK to values similar to those of control mice (Figure 7A). ADAM17 inhibition also diminished TWEAK-induced renal inflammation; decreasing the number of infiltrating inflammatory cells to control values (Figure 7B, C) and down-regulating pro-inflammatory gene expression (Figure 7D). These data clearly demonstrate that ADAM17 mediates TWEAK–EGFR pathway and downstream signalling, including ERK activation and regulation of pro-inflammatory factors. In addition, ADAM17 could be a good therapeutic target to diminish the TWEAK-induced inflammatory response in the kidney.

TWEAK induces the release of the EGFR ligands HB-EGF and TGFα via ADAM17

EGFR transactivation is mediated by the release of active EGFR ligands. Among these, HB-EGF and TGFα can be cleaved by ADAM17 [23,29]. We have found that TWEAK increased gene expression of both EGFR ligands, HB-EGF and TGFα, in the kidney and in cultured tubular epithelial cells (Figure 8A, B). ADAM17 inhibition significantly prevented ligand gene up-regulation in response to TWEAK *in vivo* and *in vitro* (Figure 8A, C). In tubular epithelial cells TWEAK, via ADAM17, also induced the release of the active ligands as evaluated by ELISA (Figure 8D). These data suggest that ADAM17 mediates the shedding of HB-EGF and TGFα in response to TWEAK stimulation.

Finally, the involvement of HB-EGF and TGFα in the TWEAK–EGFR pathway was evaluated using specific blockers of each ligand. HB-EGF inhibition, using CRM197, a non-toxic mutant of diphtheria toxin that neutralizes HB-EGF binding to EGFR [29], diminished EGFR phosphorylation caused by TWEAK (Figure 8E). TGFα blockade, using a specific neutralizing antibody, inhibited TWEAK-mediated EGFR activation (Figure 8E). Additionally, we tested whether HB-EGF or TGFα exert pro-inflammatory actions on tubular cells. Stimulation of MCT cells with the recombinant ligands HB-EGF or TGFα increased pro-inflammatory gene expression (Figure 8F). These data suggest that both ligands are involved in the activation of the EGFR pathway induced by TWEAK and, probably, in the regulation of inflammatory factors.

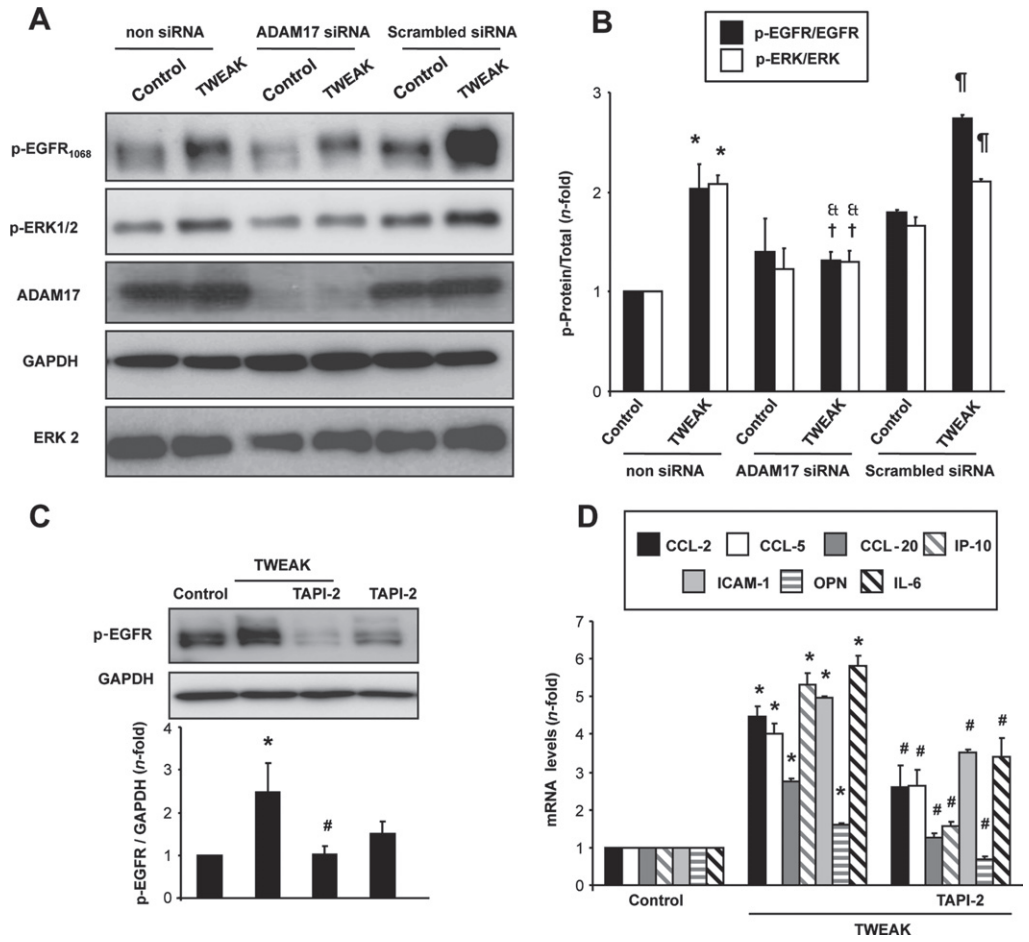

Figure 6. Inhibition of ADAM17 blocks TWEAK-mediated EGFR activation in cultured tubular epithelial cells. HK2 cells were transfected with an ADAM17, scrambled or non-siRNA, before stimulation with 100 ng/ml TWEAK for 15 min. (A) Representative western blot of phosphorylated EGFR and ERK1/2 levels, and quantification (B) expressed as mean ± SEM of three independent experiments. EGFR, ERK1/2 or GAPDH levels were used as loading control and ADAM17 as silencing control: $^{*}p < 0.05$ versus control (untreated) untransfected cells (non siRNA added). $^{¶}p < 0.05$ versus untreated scramble siRNA-transfected cells. $^{†}p < 0.05$ versus TWEAK-treated scramble siRNA-transfected cells. $^{\&t}p < 0.05$ versus TWEAK-treated untransfected cells. (C) HK2 cells were pre-incubated with the specific ADAM17 inhibitor TAPI-2 (50 μM) for 1 h; representative western blot experiment and data expressed as mean ± SEM of four experiments. (D) ADAM17 inhibition prevents TWEAK-induced up-regulation of pro-inflammatory genes. MCTs were pre-incubated with TAPI-2 and then stimulated with 100 ng/ml TWEAK for 6 h; figure shows gene expression, evaluated by real-time RT–PCR and expressed as mean ± SEM of four experiments: $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus TWEAK

NF-κB activation induced by TWEAK is not regulated by EGFR transactivation

NF-κB is an important transcription factor involved in the regulation of many pro-inflammatory genes [35]. TWEAK-injected mice presented increased nuclear levels of p65 (RelA) NF-κB subunit compared to controls, showing that TWEAK activates the NF-κB pathway in the kidney, as previously described [13]. EGFR kinase or ADAM17 inhibition did not modify p65 NF-κB nuclear levels in TWEAK-injected mice (Figure 9A, B). Moreover, *in vitro* studies using gene silencing of EGFR or ADAM17, as well as pharmacological inhibitors, showed that the EGFR–ADAM17 signalling is not involved in the activation of the NF-κB pathway caused by TWEAK (Figure 9C–F). In tubular cells, TNFα promotes EGFR transactivation via ADAM17 to regulate cell growth, although the ligand involved has not been identified [36]. In this study, TNFα-mediated NF-κB activation was independent of EGFR transactivation [36], showing a similar response to TWEAK (Figure 10).

Discussion

In this study we have identified EGFR transactivation as a novel pathway for TWEAK–Fn14-induced kidney inflammation. Several studies have shown that TWEAK elicits renal responses through Fn14 engagement [10]. Our *in vitro* studies demonstrate that *Fn14* gene silencing inhibited TWEAK-induced EGFR phosphorylation. Since Fn14 lacks tyrosine kinase activity, the EGFR pathway is activated by intracellular mechanisms that lead to EGFR transactivation. We describe here that TWEAK engages Fn14 to activate ADAM17, which releases the mature ligands HB-EGF and TGFα that, in turn, transactivate EGFR (Figure 10).

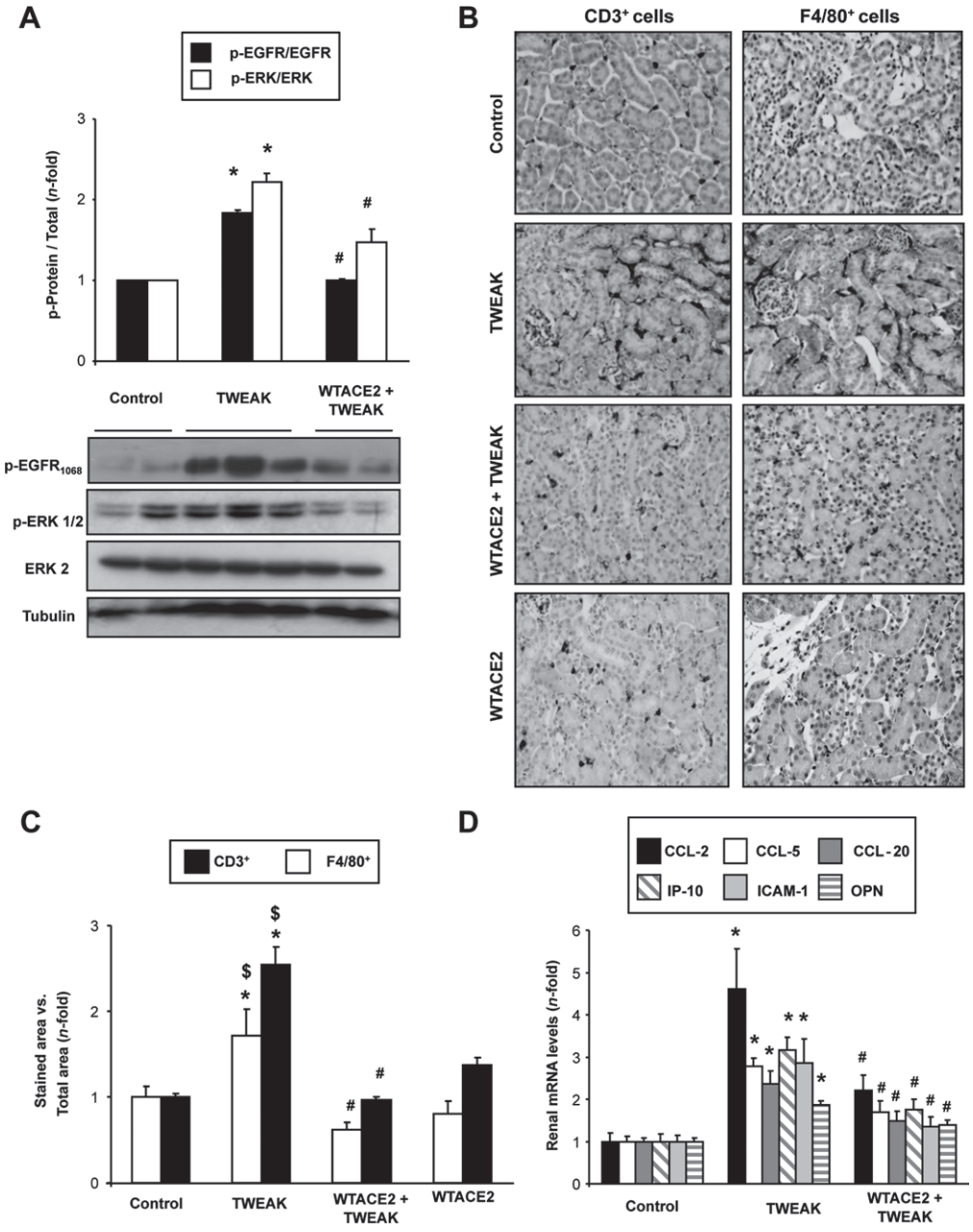

Figure 7. ADAM17 inhibition by WTACE2 ameliorates TWEAK-induced renal damage. Mice were treated with WTACE2 (ADAM17 inhibitor, 100 mg/kg/day), starting 24 h before TWEAK (0.5 μg/mouse) or saline administration, and sacrificed 24 h later. (A) EGFR/ERK activation was assessed by western blot, using an antibody against p-$EGFR_{1068}$ or p-ERK1/2. WTACE2 inhibits TWEAK-induced inflammatory cell infiltration in the kidney. (B) Representative immunostaining of inflammatory infiltrates and (C) quantification. (D) Gene expression levels were evaluated in total renal extracts by real-time PCR. All data are expressed as mean ± SEM of six to eight animals/group: $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus TWEAK; $^{\$}p < 0.05$ versus WTACE2 alone

Most studies have shown an important role for the family of membrane-anchored disintegrin-metalloproteases, ADAMs, in EGFR transactivation. There are multiple ADAMs, with ADAM17 being the most relevant in the kidney. ADAM17 mRNA is constitutively expressed in normal adult human kidneys and is increased in pathological conditions [37], as observed here in response to systemic TWEAK administration in mice. Our *in vitro* studies in tubular epithelial cells clearly demonstrate that TWEAK increased the ADAM17-mediated release of TGFα and HB-EGF, leading to EGFR transactivation. In mice, ADAM17-mediated TGFα shedding is involved in Angiotensin II-induced experimental renal fibrosis [19]. The functional consequences of TWEAK/ADAM17/EGFR activation in the kidney include activation of the key downstream intracellular signalling molecule ERK1/2, and the regulation of inflammation (Figure 10). Our experimental data demonstrate that TWEAK activates the EGFR pathway in the kidney, mainly in tubular epithelial cells. In these cells, *in vivo* and *in vitro* TWEAK induced EGFR phosphorylation on tyrosines 1068 and 1173, which have previously been associated with MAPK

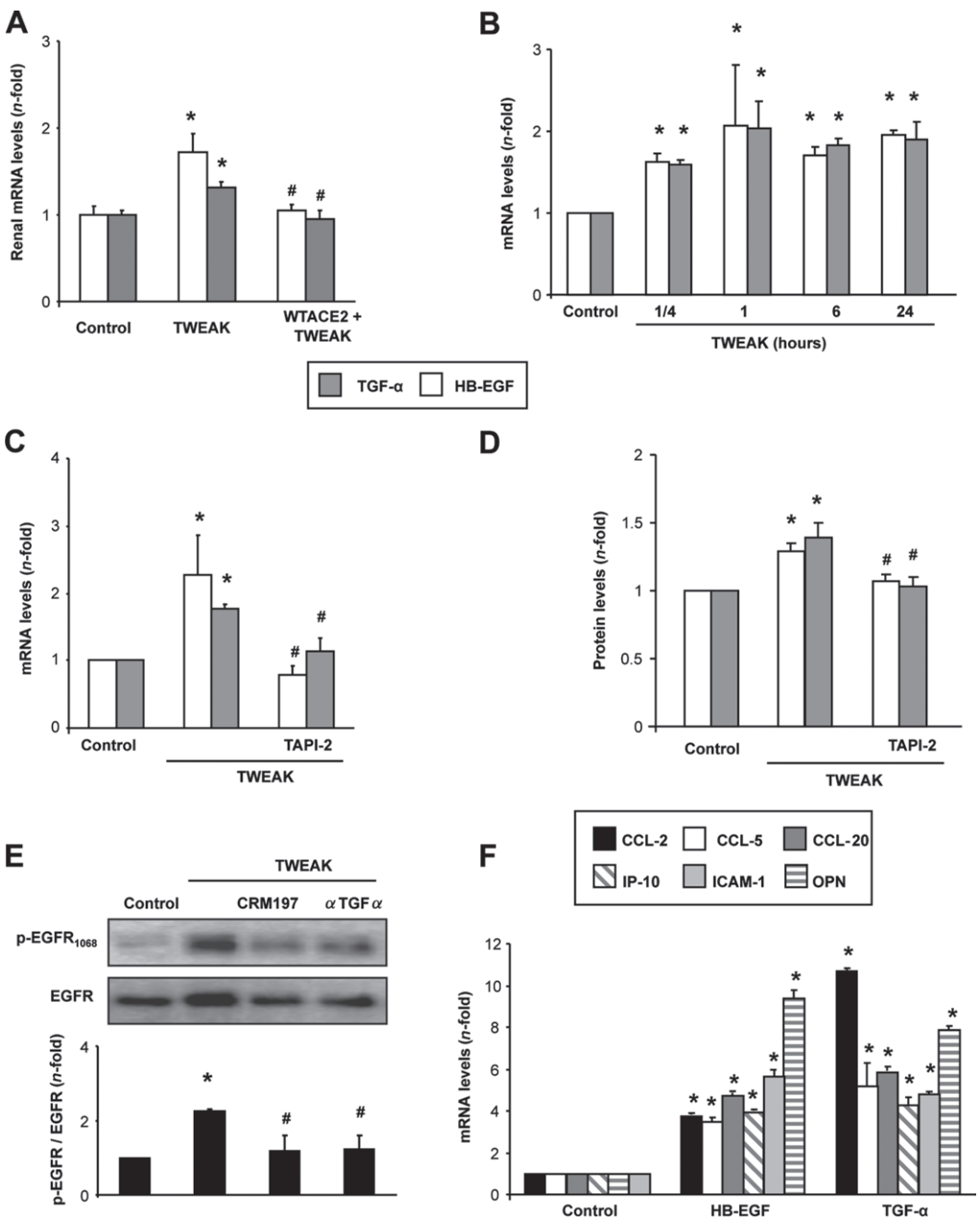

Figure 8. TWEAK increases gene expression of the EGFR ligands HB-EGF and TGFα in the kidney and in cultured mouse tubular epithelial cells. (A) In total renal extracts from mice injected with TWEAK or saline (control), or pretreated with WTACE2 (ADAM17 inhibitor), gene expression was evaluated by real-time RT–PCR and expressed as mean ± SEM of 8–10 animals/group: $^{*}p < 0.05$ versus control. (B) Murine tubular epithelial cells were stimulated with 100 ng/ml TWEAK for increasing times. ADAM17 mediates TWEAK-induced gene and protein release of HB-EGF and TGFα. Cells were pretreated for 1 h with the ADAM17 inhibitor TAPI-2 (50 μM) before TWEAK stimulation for 15 min (gene; C) or 1 h (protein; D). HB-EGF and TGFα were determined by ELISA in conditioned medium. (E) TWEAK, via HB-EGF and TGFα release, transactivates EGFR. HK2 cells were pretreated with the HB-EGF pharmacological inhibitor CRM197 (10 μM) or with a neutralizing antibody against TGFα (2.5 μg/ml) for 1 h, before stimulation with 100 ng/ml TWEAK for 15 min. (F) HB-EGF and TGFα up-regulate pro-inflammatory genes. MCTs were incubated with recombinant 100 ng/ml HB-EGF or 20 ng/ml TGFα for 6 h; figures show data expressed as mean ± SEM of three or four independent experiments: $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus TWEAK

cascade [32,33]. Experimental studies have shown that TWEAK is an important mediator of acute kidney injury through the modulation of the inflammatory response [10,13]. Now we have found that blockade of the EGFR–ADAM17 pathway, using the EGFR kinase inhibitor Erlotinib or the ADAM17 inhibitor WTACE, prevented TWEAK-mediated renal inflammation and ERK activation. Our *in vitro* studies also demonstrate the role of the ADAM17–EGFR–ERK pathway in the regulation of pro-inflammatory factor expression. Other studies in different settings support the involvement of ADAM17 in inflammation [20]. Targeting ADAM17 or ADAM10 by pharmacological inhibition or gene knockout attenuates the inflammatory response in animal models of vascular damage, including hypertension and atherosclerosis [38–40]. There are few studies evaluating the effect of EGF ligands in the kidney. HB-EGF-deficient mice were protected from

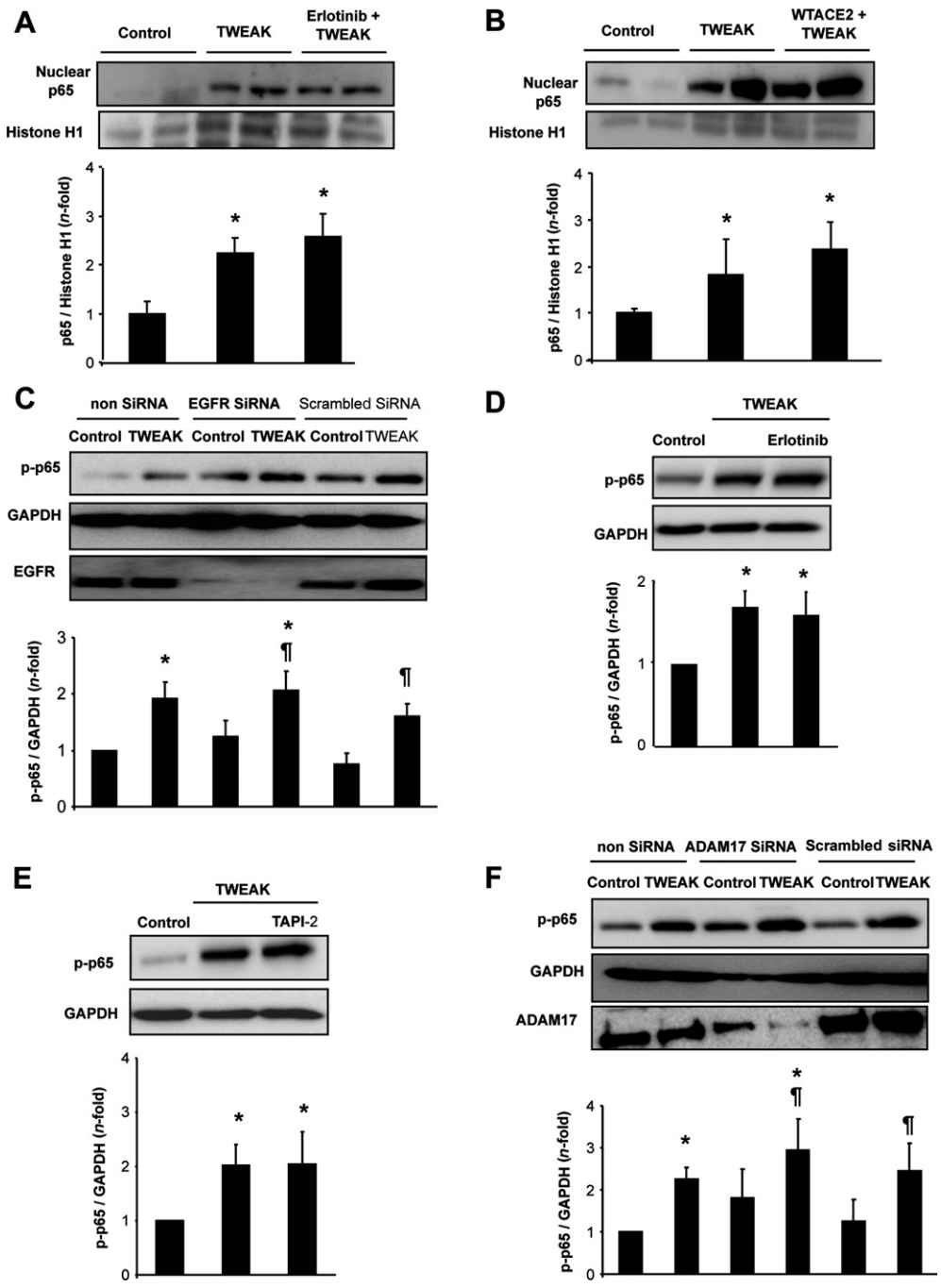

Figure 9. NF-κB activation is not involved in the TWEAK–ADAM17–EGFR pathway. (A, B) Inhibition of EGFR kinase or ADAM17 did not modify TWEAK-induced NF-κB activation in the kidney. Activation of renal NF-κB was determined by evaluation of p65 nuclear levels by western blot. (A, B) Two representative mice from each group and the quantification expressed as mean ± SEM of 8–10 mice/group. EGFR blockade did not modify TWEAK-induced NF-κB activation in cultured human tubular epithelial cells. Cells were transfected with EGFR, scrambled or non siRNA (C) or pre-incubated for 1 h with 10 μM Erlotinib (D) before stimulation with 100 ng/ml TWEAK for 15 min. ADAM17 blockade did not modify TWEAK-induced NF-κB activation. HK2 cells were pre-incubated for 1 h with TAPI-2 (E) or transfected with ADAM17, scrambled or non-siRNA (F), before stimulation with 100 ng/ml TWEAK for 15 min; figures show a representative western blot and data as mean ± SEM of four experiments: $^{*}p < 0.05$ versus the corresponding control; $^{¶}p < 0.05$ versus untreated scramble siRNA-transfected cells

inflammatory renal infiltrates and albuminuria prior to the development of marked renal cell proliferation [41]. Several studies have demonstrated that HB-EGF regulates cell proliferation [42,43] and podocyte damage in rapidly progressive glomerulonephritis [41]. TGFα participates in renal fibrosis [19]. We have found that *in vitro* stimulation with recombinant HB-EGF or TGFα up-regulated pro-inflammatory genes in tubular cells, suggesting that EGFR activation by both ligands could be involved in renal inflammation.

Several experimental studies have demonstrated that EGFR blockade could be an important tool in renal disease, mainly through regulation of cell proliferation and fibrosis [43]. EGFR blockade diminished cystogenesis,

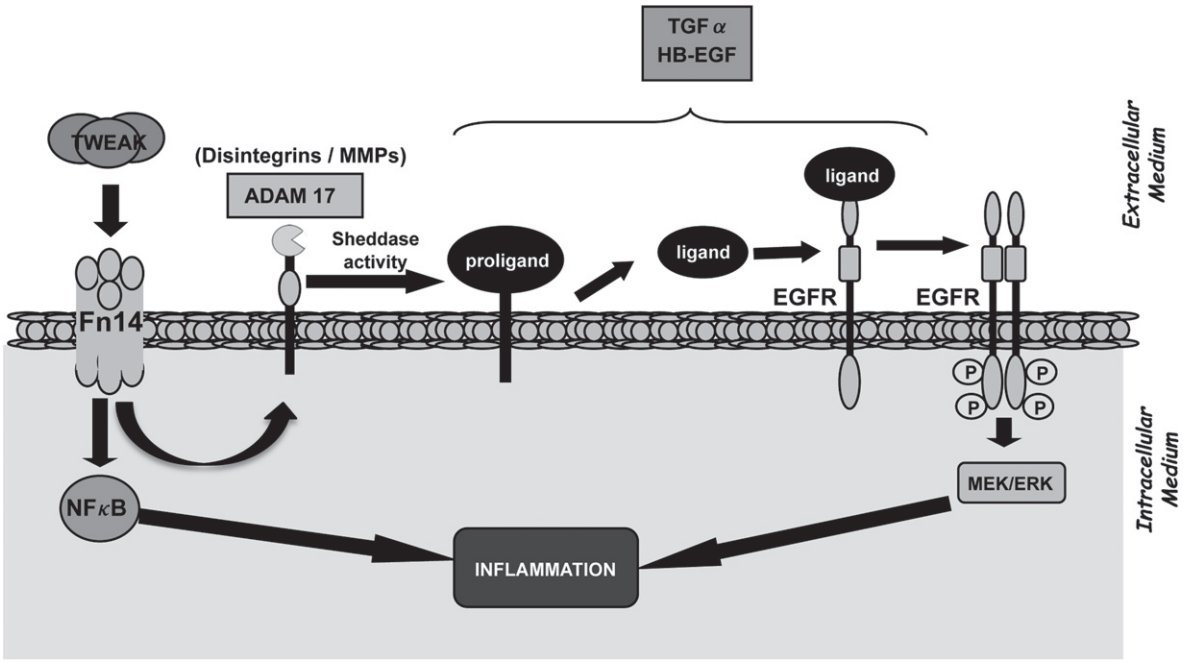

Figure 10. Mechanisms involved in TWEAK-induced renal inflammation. TWEAK engagement of Fn14 results in both the activation of the NF-κB pathway and the recruitment of ADAM17 sheddase activity, two independent responses linked to renal inflammation. We demonstrate here that TWEAK via ADAM17 can release the membrane-bound EGFR ligands, TGFα and HB-EGF, leading to EGFR transactivation, and subsequent downstream responses, including ERK/MEK activation and pro-inflammatory gene over-expression

as demonstrated in *waved-2*-mutated mice [44]. Injection of a mouse–human chimeric anti-EGFR antibody inhibited the growth of human renal-cell carcinoma xenografts in nude mice [45]. In two experimental murine models of renal injury, transgenic expression of a truncated dominant negative EGFR in proximal tubular cells resulted in a marked diminution in tubular cell proliferation, interstitial collagen accumulation and mononuclear cell infiltration [18]. In streptozotocin-induced diabetic nephropathy, an EGFR inhibitor reduced tubular epithelial cell proliferation [46]. In this regard, functional EGFR signalling was involved in tubular repair during ischaemia–reperfusion injury [47]. Our data extend previous observations to show the involvement of EGFR in the regulation of the inflammatory response, disclosing a novel therapeutic target for inflammatory kidney diseases. It is conceivable that the overall therapeutic effect of EGFR targeting will depend on the relative contribution of inflammation versus the need of proliferative repair in each type or stage of kidney injury.

TWEAK regulates cell proliferation, differentiation or apoptosis, depending on the cell type and context [4,9,48,49]. TWEAK induces apoptosis in tumour cell lines and monocytes [50], while it increases renal tubular cell proliferation [14]. In astrocytes, long-term TWEAK-mediated cell proliferation is regulated by ERK/EGFR transactivation via TGFα [51]. Several experimental studies have shown the role of TWEAK in cancer and clinical trials have explored the role of humanized anti-TWEAK receptor antibodies in cancer (http://clinicaltrials.gov/ct2/show/NCT00738764). Silencing ADAM17 in human renal carcinoma cell lines corrects critical features associated with cancer cells, including growth autonomy, tumour inflammation and tissue invasion [52]. Moreover, EGFR kinase inhibitors are also one of the therapeutic strategies with beneficial results in cancer [53]. Therefore, our findings that TWEAK transactivates EGFR via ADAM17 activation could potentially be extrapolated to proliferative disorders.

In summary, our *in vivo* studies demonstrate that blockade of EGFR transactivation by either EGFR kinase or ADAM17 inhibition ameliorates experimental renal damage caused by TWEAK, preventing the up-regulation of pro-inflammatory factors and inflammatory cell infiltration. These results suggest that inhibition of EGFR transactivation could be a novel therapeutic tool for kidney inflammation caused by TNF superfamily cytokines.

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Author contributions

SR-M contributed to the design of the experiments, acquisition, analysis and interpretation of all data and drafting of the manuscript; JLM-P and ABS contributed to acquisition of data and participated in the development of the mouse models and data analysis; AMR contributed to acquisition of some data, critical review of the manuscript and financial support of the study; SE contributed to design of the experiments (ADAM17), analysis of the results and critical review of the manuscript; DB contributed to analysis of the results, data of *in vivo* WTACE and critical review of the manuscript; JP and GK contributed to chemical synthesis of the EGFR kinase inhibitor and *in vivo* Erlotinib data; AO and JE contributed to critical review of the manuscript and financial support of the study; MR-O contributed to the design of the experiments, analysis and interpretation of the data, drafting of the manuscript and financial support of the experiments. All the authors reviewed the manuscript and approved the final version to be published.

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5. Paricalcitol presenta efectos antiinflamatorios en el daño renal inducido por TWEAK mediante la regulación del eje ADAM17/EGFR y de la vía no canónica de NF-κB.

La deficiencia de vitamina D y de sus metabolitos activos es una situación común en la patogenia de la ERC (Valdivielso et al., 2009, Levin et al., 2006). Datos experimentales en modelos animales sugieren que la vitamina D y los VDRAs pueden revertir la progresión de la ERC a través de la prevención de la proliferación, la inflamación y la fibrosis (Mizobuchi et al., 2007, Kuhlmann et al., 2004, Panichi et al., 2001, Tan et al., 2006, Hirata et al., 2002). Previamente se ha descrito que TWEAK induce la transactivación del EGFR en el riñón regulando la inflamación renal (Rayego-Mateos et al., 2013b). En este trabajo se ha abordado el objetivo 4 de la tesis que ha consistido en estudiar si los efectos antiinflamatorios de los VDRAs son debidos a la modulación de la vía del EGFR inducida por TWEAK. Para ello se desarrolló un modelo de administración sistémica de TWEAK en ratones, tratados con el VDRA Paricalcitol. El tratamiento con paricalcitol disminuyó significativamente la fosforilación del EGFR, la sobreexpresión de factores proinflamatorios y la presencia de infiltrado inflamatorio en el riñón de los ratones inyectados con TWEAK. En células tubuloepiteliales renales en cultivo, Paricalcitol también inhibió la trasactivación del EGFR inducida por TWEAK y la inducción de factores proinflamatorios. Sin embargo, en los estudios *in vivo* e *in vitro*, Paricalcitol no inhibió la activación de la vía canónica de NF-κB inducida por TWEAK, que conlleva la fosforilación de factores como IκB-α o RelA. Bibliografía previa ha demostrado la capacidad de TWEAK de activar la vía no canónica de NF-κB (Sanz AB et al., 2010). Paricalcitol inhibió la activación de la vía no canónica de NF-κB inducida por TWEAK, caracterizada por la regulación de factores como p100/p52, IKK-α o Rel B y citoquinas como CCL19 y CCL21.

En resumen, estos datos sugieren que las acciones antinflamatorias de paricalcitol pueden ser debidas a la inhibición tanto de la ruta del EGFR como de la vía no canónica del NF-κB en respuesta a TWEAK, mostrando un nuevo e importante mecanismo de las acciones beneficiosas de los análogos de la vitamina D.

Title page

Paricalcitol exerts anti-inflammatory effects in experimental renal damage through modulation of the ADAM-17/EGFR axis and the non-canonical NF-κB pathway

Authors:

Sandra Rayego-Mateos[1], Jose Morgado-Pascual[1], Ana B Sanz[2], Jose M. Valdivielso[3], Jesus Egido[4], Alberto Ortiz[2], Marta Ruiz-Ortega[1]

[1] Cellular Biology in Renal Diseases Laboratory. Universidad Autónoma Madrid. Spain.

[2] Dialysis Unit. IIS-Fundación Jiménez Díaz-UAM. School of Medicine, UAM and IRSIN, Madrid Spain.

[3] Department of Experimental Nephrology, Universitat de Lleida/Institut de Recerca Biomèdica de Lleida, Lleida 25198, Spain.

[4] Division of Nephrology and Hypertension. IIS-Fundación Jiménez Díaz-UAM/IRSIN. Madrid Spain

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Correspondence:
Marta Ruiz-Ortega, PhD.
Cellular Biology in Renal Diseases Laboratory
Universidad Autónoma Madrid
Instituto de Investigación Sanitaria Fundación Jiménez Díaz
Avda. Reyes Católicos, 2
28040 Madrid, Spain
Tel. 34-91-5504821
Fax. 34-91-5442636
Email: mruizo@fjd.es

ABSTRACT

Chronic kidney disease is characterized by Vitamin D deficiency. Vitamin D receptor agonists (VDRAs) exert beneficial effects in renal disease and possess anti-inflammatory properties, but the underlying mechanism remains unknown. TWEAK induces EGFR transactivation regulating several processes including renal inflammation. Activation of the NF-κB pathway has been involved in inflammatory renal diseases, however, there are few studies evaluating the contribution of non-canonical NF-κB pathway to renal disease progression. Our aim was to investigate the involvement of ADAM17/EGFR axis in VDRA anti-inflammatory effects and the downstream mechanisms, with special attention to non-canonical NF-κB pathway. Preincubation with the VDRA paricalcitol inhibited TWEAK-induced EGFR transactivation and downstream mechanisms, including proinflammatory factor overexpression and inflammatory cell infiltration in the kidney. Paricalcitol prevented non-canonical NF-κB activation, as determined by IKK-α activation and RelB and NF-κB2 p100/p52 nuclear translocation and DNA binding activity. Interestingly, activation of canonical NF-κB, including IκB-α and NF-κB p65 phosphorylation, was not modified by paricalcitol in kidneys from TWEAK-injected mice. Moreover Paricalcitol inhibited the overexpression of chemokines regulated by non-canonical NF-κB activation, including CCL-21 and CCL-19. In conclusion, our data suggest that the anti-inflammatory actions of paricalcitol could depend on the inhibition of EGFR and non-canonical NF-κB pathway in response to TWEAK, identifying an important mechanism for VDRAs effects.

INTRODUCTION

One of the earliest consequences of chronic kidney disease (CKD) patients is active vitamin D deficiency[1,2]. Vitamin D receptor agonists (VDRA) decrease proteinuria, and may reduce renal damage progression and improve cardiovascular outcomes in CKD patients[1-3]. These beneficial effects are independent of serum parathyroid hormone, phosphorus, and calcium levels suggesting that vitamin D presents pleotropic actions, beyond mineral metabolism regulation[1,4]. Active vitamin D (1,25–dihydroxy vitamin D(3) or calcitriol) mediates its biological effects by binding to the vitamin D receptor (VDR), which then translocates to the nuclei of target cells[1]. In experimental renal disease vitamin D or VDRAs treatment diminished fibrosis, mesangial proliferation, podocyte loss and inflammatory cell infiltration[5-10]. However, the molecular mechanism involved in the anti-inflammatory effects of vitamin D in the setting of CKD remains poorly characterized.

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a multifunctional cytokine, a member of the TNF superfamily, which binds to its cognate receptor fibroblast growth factor-inducible molecule 14 (Fn14) to elicit cellular responses[11]. TWEAK activation of Fn14 regulates several biological responses, such as proinflammatory activity, angiogenesis and cell proliferation, and, under some experimental conditions, apoptosis. Excessive or persistent TWEAK and Fn14 activation drives pathological tissue responses, leading to progressive damage and degeneration[12]. TWEAK targeting has demonstrated beneficial effects in experimental renal damage[13]. Neutralizing anti-TWEAK antibodies completed a successful phase I clinical trial and are undergoing phase II studies in lupus nephritis.

Emerging evidence suggests that blockade of epidermal growth factor receptor (EGFR) could be a therapeutic option for renal diseases. Experimental studies have shown that genetic or pharmacological EGFR blockade ameliorate renal disease progression, mainly by diminishing kidney fibrosis[14,15]. TWEAK can “trans”-activate EGFR, via “a disintegrin and metalloproteases” (ADAM-17), thus regulating renal inflammation[16]. ADAMs are membrane-spanning metalloproteases involved in cleavage of extracellular substrates (shedding), including EGF-family ligands, both constitutively and in response to regulatory stimulation [17,18]. In the kidney, ADAM17, also known as TACE, participates in the shedding of the EGFR ligands, heparin binding EGF-like growth factor (HB-EGF) and transforming growth factor-α (TGF-α) [19-22].In mice, ADAM17-mediated TGF-α shedding contributes to Angiotensin II-induced experimental renal fibrosis [15] and to experimental renal inflammation induced by systemic TWEAK administration[16].

Among the intracellular mechanisms involved in renal inflammation, NF-κB has attracted special interest. TWEAK activates the canonical NF-κB pathway to induce the expression of both soluble and membrane-bound inflammatory chemokines, including MCP-1, RANTES [23] and CXCL16 [24]. TWEAK also activates the non-canonical NF-κB pathway to induce the expression of additional chemokines, such as CCL21 and CCL19 in tubular cells[25]. Interestingly, EGFR transactivation elicited by TWEAK did not modulate classical NF-κB pathway activation[16]. There are few studies evaluating the contribution of the

non-canonical NF-κB pathway to renal disease progression. In this paper we will investigate the molecular mechanisms involved in the anti-inflammatory effects of VDRAs such as paricalcitol in renal disease. For this study, the experimental model of TWEAK-induced renal damage was used, evaluating the ADAM17/EGFR signaling axis and the non-canonical NF-κB pathway, as potential important inflammatory mechanisms.

RESULTS

The VDRA Paricalcitol diminishes TWEAK-induced renal inflammation

To investigate the *in vivo* effect of Vitamin D analogs in TWEAK-induced renal damage, mice were treated with the VDRA paricalcitol. TWEAK administration caused an inflammatory response in the kidney, as previously described[23]. In TWEAK-injected mice, paricalcitol significantly diminished the number of infiltrating monocytes/macrophages ($F4/80^+$ cells) and T lymphocytes ($CD3^+$ cells) (Figure 1A and B). Paricalcitol inhibited TWEAK-induced upregulation of proinflammatory gene expression, including cytokines (IL-6), chemokines (CCL2, CCL5) and their receptors (CCR2) (Figure 2A). Paricalcitol also blocked renal chemokine production, as observed in the case of CCL2 and CCL5 (**F**igure 2B).

The direct effect of paricalcitol on proinflammatory TWEAK actions was characterized in cultured cells. In tubular epithelial cells, preincubation with paricalcitol for 48 hours inhibited TWEAK-induced proinflammatory gene overexpression (Figure 2C). These data confirm the anti-inflammatory properties of paricalcitol, and show that VDRA diminish TWEAK-induced inflammatory responses in the kidney and in cultured renal cells.

Paricalcitol modulates TWEAK-induced activation of the ADAM-17/EGFR axis

TWEAK induced EGFR transactivation regulates the kidney inflammatory response[23]. Paricalcitol diminished renal EGFR phosphorylation levels in TWEAK-injected mice to values similar to control mice, as shown by western blot (Figure 3A). Thus, paricalcitol inhibited TWEAK-induced EGFR transactivation. By immunohistochemistry we localized EGFR pathway activation to tubular cells and this was abolished in paricalcitol-treated mice (Figure 3B and C). Moreover, in cultured tubular epithelial cells, preincubation with paricalcitol for 48 hours also reduced EGFR phosphorylation in response to TWEAK (Figure 3D).

ADAM17 regulates EGFR transactivation induced by several factors, including Angiotensin II and TWEAK[16,26]. In TWEAK-injected mice ADAM-17 was upregulated at the gene and protein expression levels (figure 4A and B), as observed in other models of renal damage [27]. Paricalcitol downregulated ADAM-17 gene and protein levels to control values (Figure 4A and B). In cultured tubular epithelial cells, paricalcitol also reduced TWEAK-induced ADAM-17 gene overexpression (Figure 4C).

During renal damage VDR expression is down-regulated[1,3]. Interestingly, in mice and in cultured tubular epithelial cells TWEAK down-regulated VDR gene expression, but Paricalcitol pre-treatment

restored VDR expression (Figure 5A and B). These data indicates that modulation of VDR expression may be a key effector of the beneficial effects of paricalcitol.

Finally, we have investigated whether paricalcitol modulates intracellular signaling downstream of EGFR. EGFR phosphorylation on tyrosine 1068 and 1173 is involved in triggering ERK signalling[28,29], as we have previously described in response to TWEAK administration[23]. Paricalcitol also blocked ERK activation elicited by TWEAK *in vivo* and *in vitro* (Figure 6A and B).

Paricalcitol inhibited TWEAK-induced non-canonical NF-κB activation but did not modulate canonical NF-κB activation in vivo and in vitro

TWEAK activates both the canonical and the non-canonical NF-κB pathway in the kidney and in cultured renal cells [25]. Next, we investigated whether the anti-inflammatory actions of paricalcitol were mediated by modulation of canonical or non-canonical NF-κB activation.

One of the earliest events in canonical NF-κB pathway activation is phosphorylation of IκBα and the p65 NF-κB subunit, allowing the subsequent translocation of active p65 NF-κB complexes to the nucleus[30]. In TWEAK-injected mice renal IκBα phosphorylation was higher than in controls, but this was not normalized by paricalcitol (Figure 7A). TWEAK also caused p65 NF-κB phosphorylation and nuclear traslocation. Again, these changes in p65 were not modified by paricalcitol in mice (Figure 7B and C). Moreover, *in vitro* studies showed that paricalcitol did not modulate TWEAK-induced canonical NF-κB pathway activation, as assessed by the evaluation of IκBα and p65 phosphorylation and subcellular location (Figure 8A and B). These data clearly indicates that paricalcitol does not regulate TWEAK-induced canonical NF-κB pathway activation.

Activation of the non-canonical NF-κB pathway is characterized by activation of NF-κB-inducing kinase (NIK, MAP3K14) that triggers IKK-α phosphorylation and NF-κB2 p100 processing to NF-κB2 p52 which forms heterodimers with RelB that traslocate to the nucleus to regulate gene transcription. Components of the non-canonical NF-κB pathway were predominantly located in tubular cells[30]. In cultured tubulo-epithelial cells, paricalcitol diminished TWEAK-induced IKK-α phosphorylation and NIK upregulation (Figure 9A). Moreover, paricalcitol also inhibited TWEAK-induced changes in the NF-κB2 p100/p52 ratio and nuclear p52 and RelB localization (Figure 9B). *In vivo*, TWEAK activated the non-canonical NF-κB pathway, as shown by increased renal levels of NF-κB2 p52 (figure 10A) and by the nuclear localization of NF-κB2 p52/RelB, mainly observed in tubular cells (Figure 10B). Paricalcitol blocked the nuclear traslocation of both NF-κB2 p52 and RelB (figure 10B), and significantly inhibited NF-κB2 p52 DNA binding activity, as evaluated by ELISA (Figure 9C). These data clearly demonstrated that paricalcitol inhibited TWEAK-induced non-canonical NF-κB pathway activation.

Paricalcitol inhibited non-canonical NF-κB pathway-regulated genes

Several genes are regulated by TWEAK via the non-canonical NF-κB pathway, including CCL19 and CCL21[25]. Paricalcitol inhibited renal gene expression of both chemokines (figure 11A). Moreover,

paricalcitol also diminished renal CCL21 protein levels (Figure 11B, C and D). Similar results in gene expression were found *in vitro* (Figure 11E).

DISCUSSION

Several lines of evidence suggest an anti-inflammatory activity of vitamin D in CKD[6]. Studies in experimental renal damage have found that vitamin D or other VDRAs reduce inflammatory cell infiltration[6-9]. However, the mechanisms involved in these anti-inflammatory effects are not fully understood. In experimental TWEAK-mediated renal damage, we have found that paricalcitol inhibited renal inflammation and downregulated proinflammatory mediators, through inhibition of EGFR/ADAM17 signaling, including downstream ERK activation. Moreover, the anti-inflammatory actions of paricalcitol could depend on the inhibition of non-canonical NF-κB activation in response to TWEAK, thus identifying an important novel mechanism of VDRAs action.

Earlier studies showed a relation between Vitamin D and EGFR binding and regulation of EGFR target gene expression [33-36] Moreover, Vitamin D or VDRAs decrease EGFR-induced cell proliferation through changes in EGFR membrane trafficking and down-regulation of EGFR growth signaling [1,37]. Recent studies suggest that this antiproliferative effects could be mediated by the modulation of the TGF-α/EGFR autocrine growth loop[38]. We previously described that TWEAK induced EGFR transactivation via ADAM17 in the kidney[16]. Now, using this experimental model of TWEAK-mediated renal inflammation, we have found that paricalcitol inhibited TWEAK-induced EGFR phosphorylation. Moreover, in cultured tubular cells paricalcitol also inhibited TWEAK-induced EGFR and ERK activation, suggesting that the antinflammatory actions of paricalcitol could be due to modulation of EGFR pathway. In monocytes/macrophages vitamin D inhibits LPS-induced cytokine production by upregulating MAPK phosphatase-1 that inactivates p38 and JNK[32]. Our in vivo and in vitro data identify the EGFR/ERK pathway as a novel target of VDRAs actions.

Most studies have shown an important role for the family of membrane-anchored disintegrin-metalloproteases, ADAMs, in EGFR transactivation. Among the multiple known ADAMs, ADAM17 is the most relevant in the kidney. ADAM17 mRNA is constitutively expressed in normal adult human kidneys and is increased in pathological conditions[39]. In experimental TWEAK-induced inflammation, ADAM17 renal levels were upregulated and paricalcitol prevented this upregulation. VDRAs increased VDR expression. In TWEAK-injected mice, renal VDR gene expression was reduced, and this was prevented by paricalcitol, as previously observed in cultured tubular epithelial cells[40]. These data suggest that paricalcitol effects could be due to modulation of VDR levels.

NF-κB is a key transcription factor regulating renal inflammation. Interestingly, EGFR transactivation elicited by TWEAK did not modulate the canonical NF-κB activation pathway [16]. Many experimental studies have shown that blockade of canonical NF-κB activation, using inhibitors such as parthenolide or sn50 peptides, inhibited experimental inflammation, including kidney inflammation[41-45]. However, the role of non-canonical NF-κB activation in renal disease remains poorly understood. TWEAK is one of the few cytokines that can activate both canonical and non-canonical NF-κB pathways

[16,45-48]. We have found that paricalcitol only inhibited TWEAK-induced non-canonical NF-κB pathway activation without interfering with canonical NF-κB activation, both in the kidney and in cultured renal cells. Several reports in cultured cells suggested that vitamin D inhibits the canonical NF-κB pathway, by increasing IκB-α phosphorylation and reducing NF-κB p65 nuclear translocation in mesangial cells, mouse embryonic fibroblasts, and pancreatic islet cells[49-51]. However, in experimental unilateral ureteral obstruction, paricalcitol did not reduce canonical NF-κB pathway activation [52]. Despite this, in unilateral ureteral obstruction, as in TWEAK-injected mice, paricalcitol downregulated many canonical NF-κB target proinflammatory genes, such as MCP-1. Under some circumstances, paricalcitol decreased RANTES mRNA expression by abolishing p65 binding to the RANTES promoter, through formation of VDR/p65 complexes that inhibit the ability of p65 to trans-activate gene transcription[52]. Most of these genes are regulated by MAPKs activation[53], as we have previously demonstrated in the case of MCP-1[54] Our results showing that paricalcitol inhibited EGFR/ERK signaling could explain the inhibition of the expression of some of these proinflammatory genes, independently of canonical NF-κB activation.

TWEAK activates the non-canonical NF-κB pathway requiring NIK, NF-κB2 p100 processing and RelB expression, leading the synthesis of cytokines, such as CCL21 and CCL19[47]. Few studies have analyzed the role of NF-κB2 in renal damage. Blockade of RelB protects against lethal renal ischemia[55]. RelB blockade has been associated with upregulation of SNAIL and RhoGTPasas in a transcriptomic analysis[54]. In cultured proximal tubular cells, RelB lentiviral silencers reduced apoptosis induced by the combination of TNF-α and cisplatin, associated with phenotype changes related to epithelial to mesenchymal transition[56]. We have found that paricalcitol inhibited TWEAK-induced non-canonical NF-κB pathway activation. In cultured tubular epithelial cells, paricalcitol inhibited IKK-α phosphorylation and reduced NIK, NF-κB2 p52 and RelB levels in response to TWEAK. Moreover, paricalcitol reduced NF-κB2 p52 and RelB accumulation in the kidney of TWEAK-injected mice. Non-canonical NF-κB activation leads to transcription of a set of genes different from those regulated by canonical NF-κB activation, that include chemokines as CCL-21, CCL-19, CXCL13, CXCL12 and BAFF[57]. Studies in NF-κB2 knockout mice have demonstrated that those genes require NF-κB2 to be expressed[58]. We have found that paricalcitol reduced renal CCL21 and CCL19 gene expression in response to TWEAK, suggesting that VDRAs anti-inflammatory effects could be mediated by the modulation of NF-κB2-controlled genes.

In conclusion, we now demonstrated here that the beneficial anti-inflammatory effects of VDRAs such as paricalcitol in renal disease could be explained by inhibition of TWEAK-mediated proinflammatory factor overexpression through modulation of the ADAM17/EGFR signaling axis and dampening of downstream mechanisms, including ERK and non-canonical NF-κB activation. Our data add novel information about mechanisms involved in the well-known anti-inflammatory properties of VDRAs and may contribute to better design of future clinical trials.

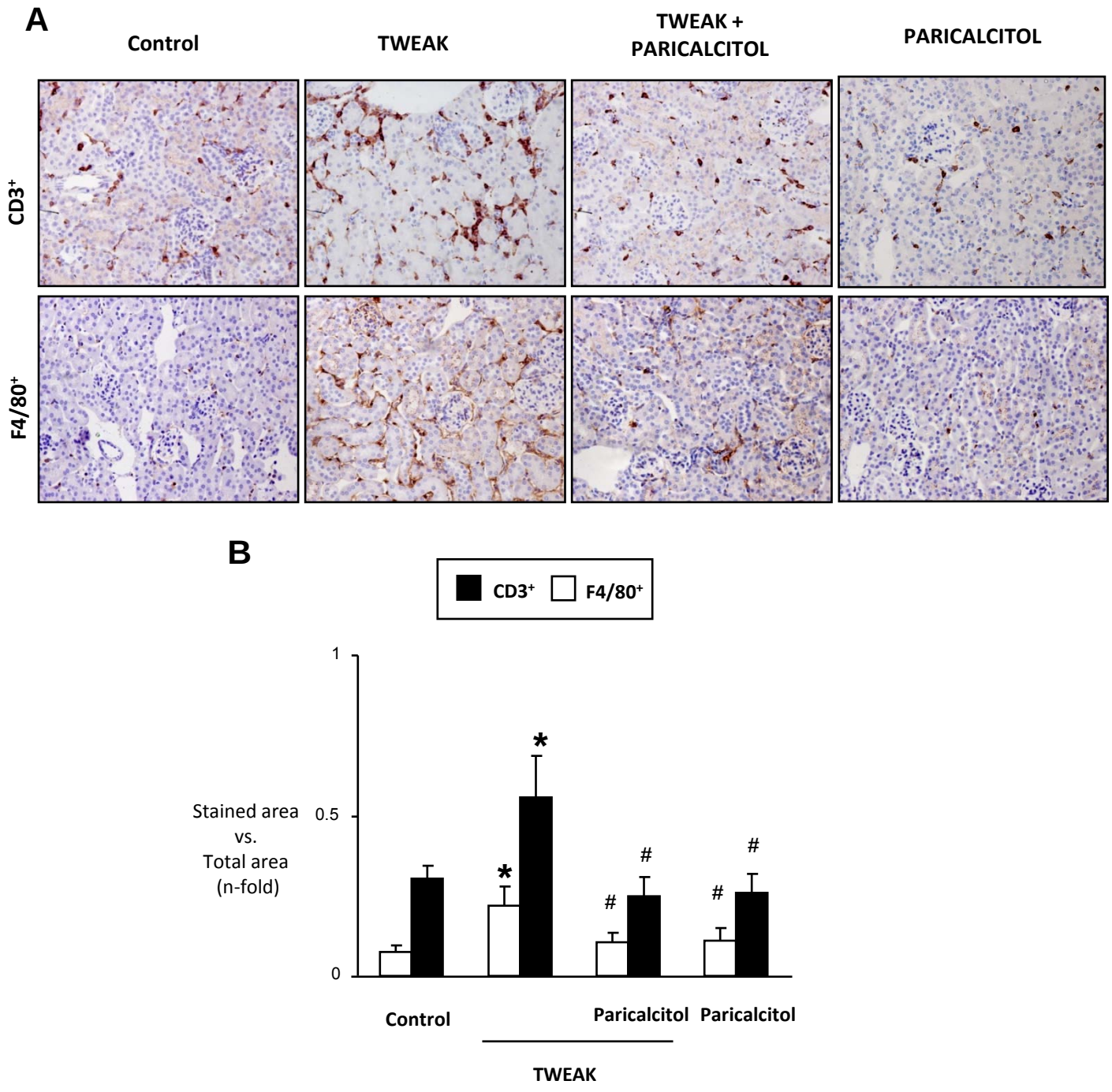

Figure 1. Paricalcitol ameliorates TWEAK-induced renal injury. C57BL/6 mice were i.p. injected with recombinant TWEAK (0.5 μg/mouse) or vehicle (saline) and sacrificed 24 hours later. Some mice were also treated with Paricalcitol (Vitamin D analog, 25 μg/kg/day), starting 48 hours before TWEAK administration. Paricalcitol inhibits TWEAK-induced inflammatory cell infiltration in the kidney to values similar to control mice. In paraffin-embedded kidney sections, immunohistochemistry using anti-F4/80 and anti-CD3 identified monocyte/macrophages and T lymphocytes, respectively. (**A)** Representative animals from each group. Magnification 200X. **(B)** Staining area quantification expressed as mean ± SEM of 8-10 animals per group. *p<0.05 vs control. # p<0.05 vs TWEAK.

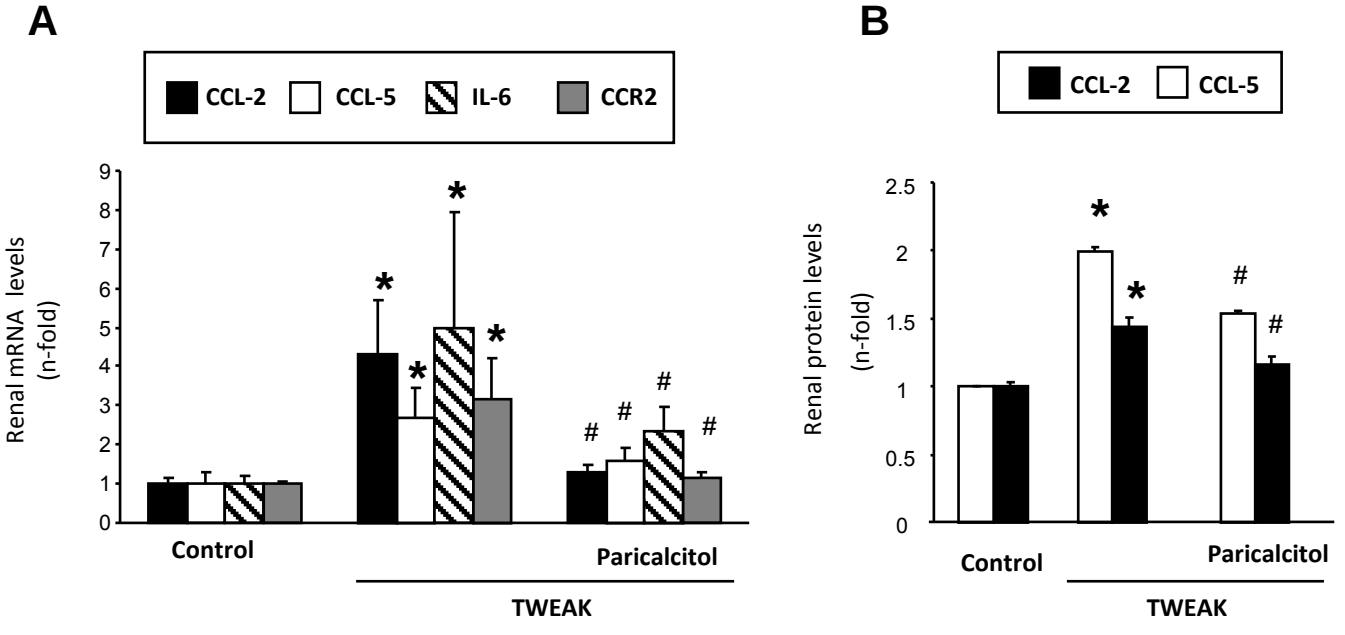

Figure 2. Paricalcitol inhibits TWEAK-induced upregulation of proinflammatory gene expression. A, B) Kidneys. RNA was obtained from total renal extracts. Proinflammatory gene expression was determined by Real Time PCR (A) and protein levels by ELISA (B). Data are expressed as mean ± SEM of 8-10 animals per group. *p<0.05 vs control. # p<0.05 vs TWEAK. C. Cultured murine tubular epithelial cells. Murine tubular epithelial cells (MCTs) were pre-incubated for 48 hours with 15 μmol/L paricalcitol before stimulation with 100 ng/mL TWEAK for 6 hours. Gene expression levels are expressed as mean ± SEM of 4 experiments. *p<0.05 vs control. # p<0.05 vs TWEAK.

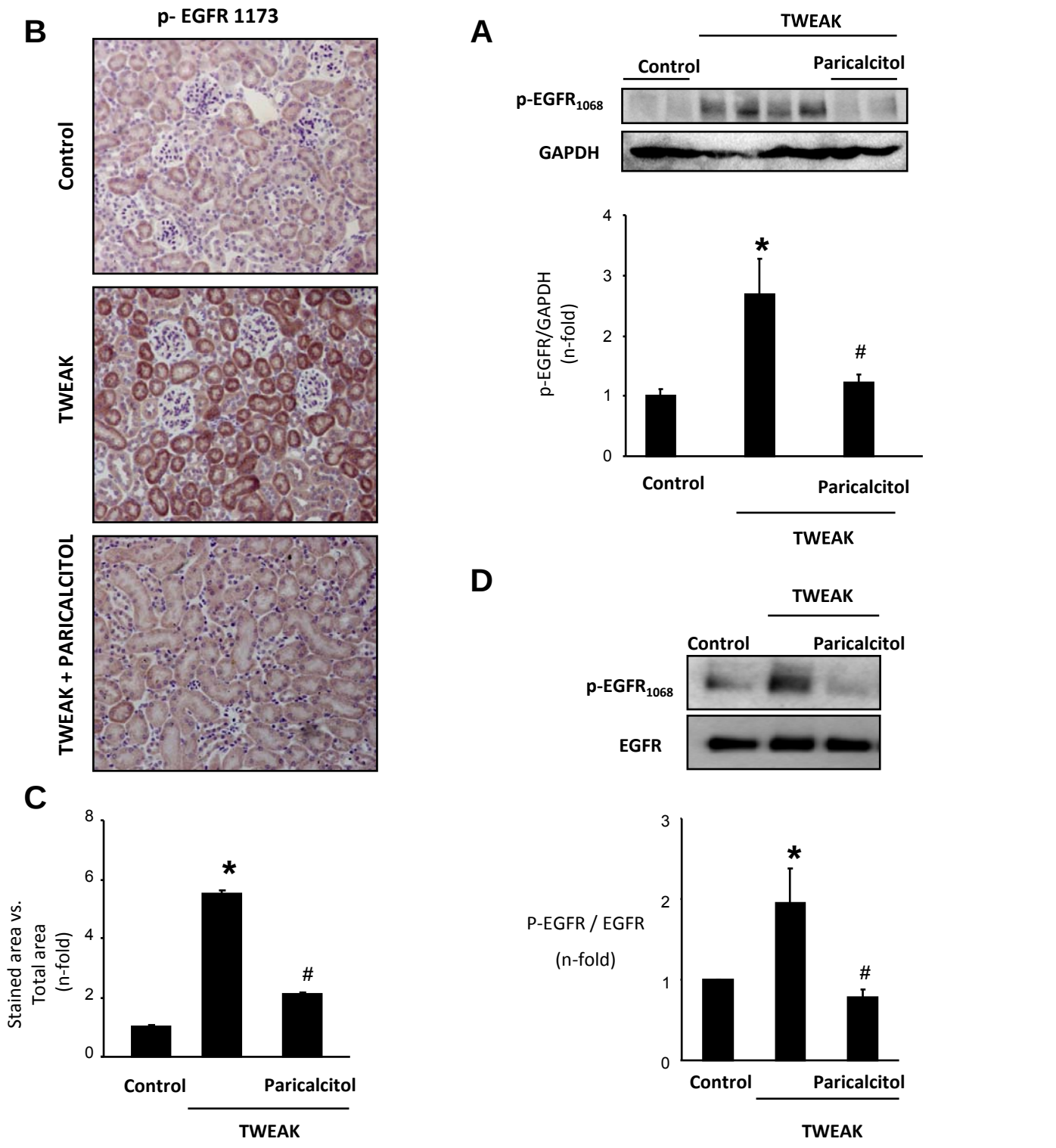

Figure 3. Paricalcitol inhibits TWEAK-induced EGFR activation in the kidney. (A) EGFR activation was assessed as EGFR phosphorylation by Western blot in total kidney protein extracts using an antibody against tyrosine 1068 phosphorylated-EGFR (p-EGFR1068). GAPDH levels were used as loading control. Representative western blot and quantitation. (B,C) Immunohistochemistry of active EGFR (using an antibody against EGFR phoshorylated on tyrosine 1173, p-EGFR1173) showed a positive immunostaining mainly in tubular epithelial cells. Figure B shows representative mice of each group and C the quantification of the data. Mean ± SEM of 8-10 mice per group. *p<0.05 vs control. # p<0.05 vs TWEAK. (D) Human tubular cells were pretreated with paricalcitol 48 hours prior to TWEAK stimulation (100 ng/ml) for 15 min. EGFR phosphorylation was evaluated by Western blot using an antibody against p-EGFR1068. GAPDH levels were used as loading controls. Data are expressed as mean ± SEM of 4 experiments. *p<0.05 vs control. # p<0.05 vs TWEAK.

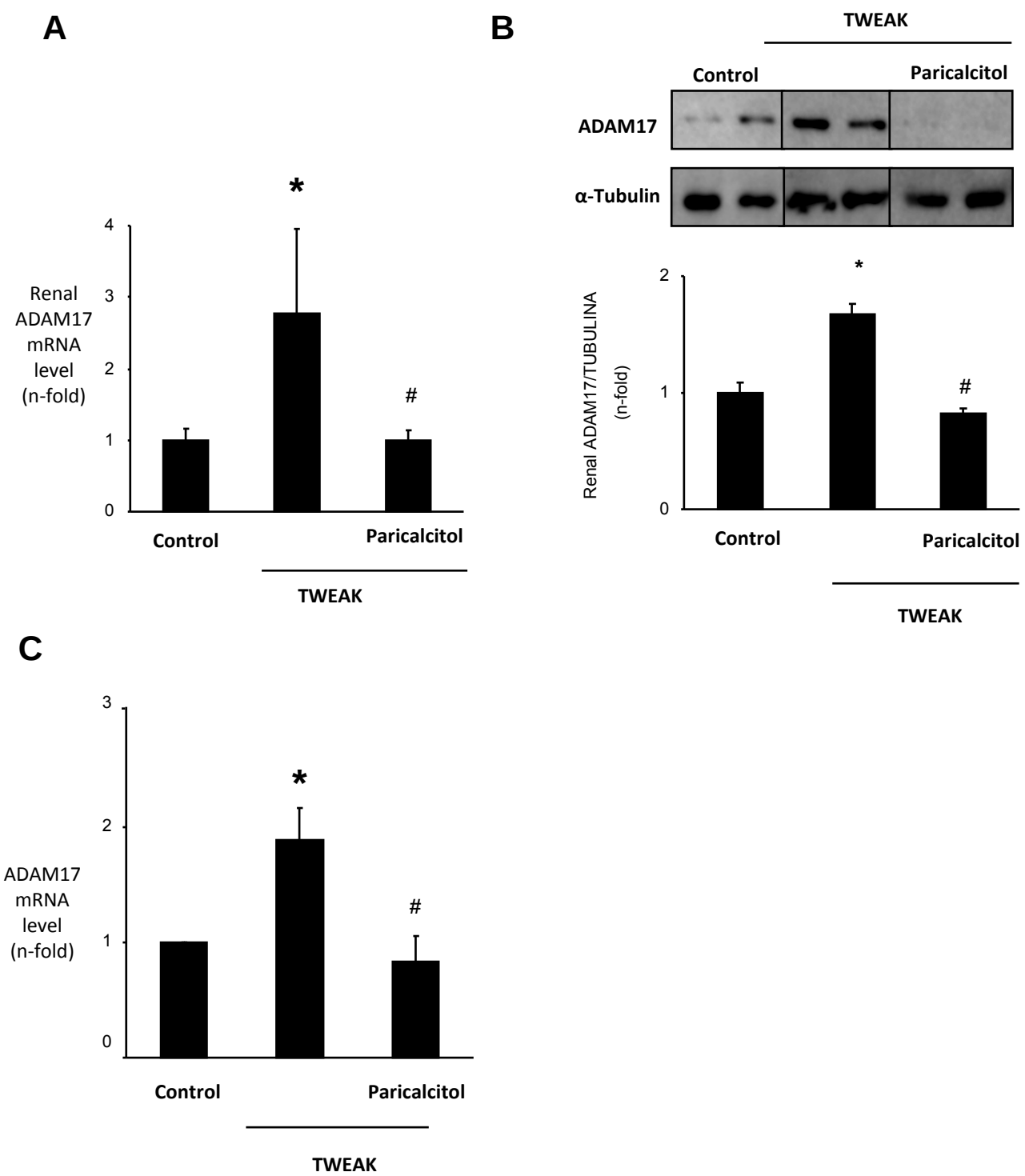

Figure 4. Paricalcitol modulates TWEAK-induced ADAM17 expression in the kidney and in cultured tubular epithelial cells. Total kidney was used for RNA and protein isolation and ADAM17 gene expression was evaluated by real time PCR (A) and protein levels by Western Blot (B). α-Tubulin was used as loading control. Data expressed as mean ± SEM of 8-10 mice per group. *p<0.05 vs control. # p<0.05 vs TWEAK. (C) Murine tubular epithelial cells (MCTs) were pre-incubated for 48 hours with 15 μmol/L paricalcitol before stimulation with 100 ng/mL TWEAK for 6 hours. Data are expressed as mean ± SEM of 4 experiments. *p<0.05 vs control. # p<0.05 vs TWEAK.

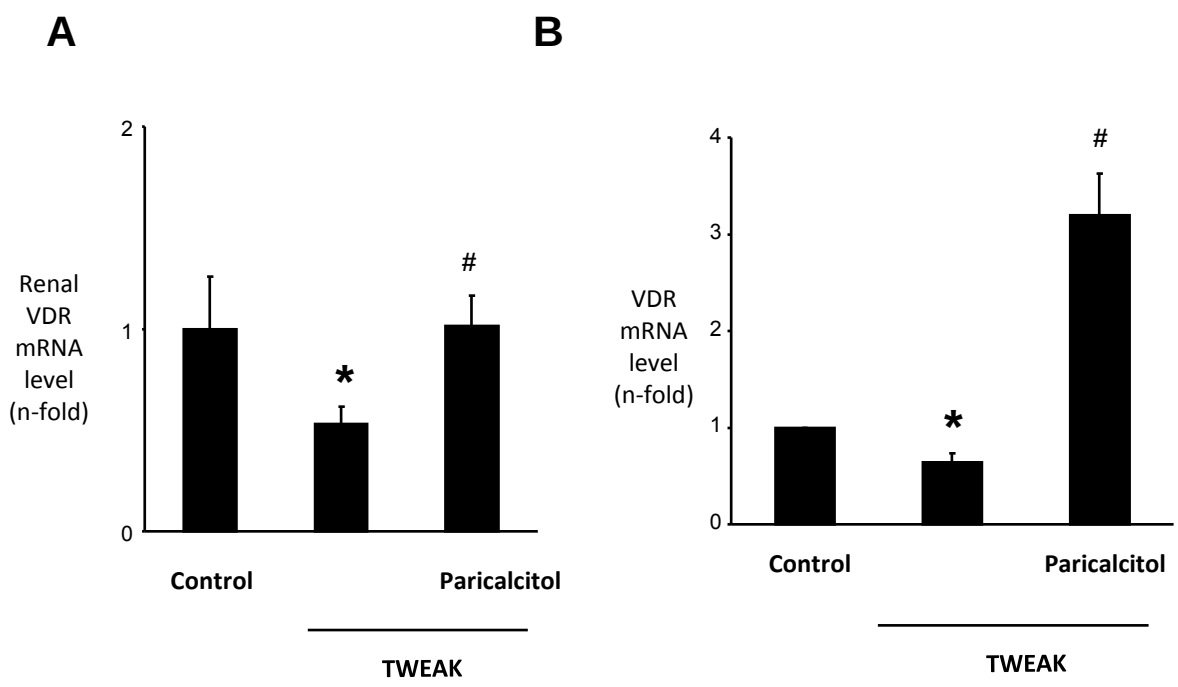

Figure 5. Paricalcitol prevents TWEAK-induced downregulation of VDR gene expression in vivo and in vitro. (A) VDR gene expression was evaluated by real time PCR in renal samples from mice and (B) cells treated with TWEAK and/or paricalcitol. Data are expressed as mean ± SEM of 8-10 animals per group or 4 independent in vitro experiments. *p<0.05 vs control. # p<0.05 vs TWEAK.

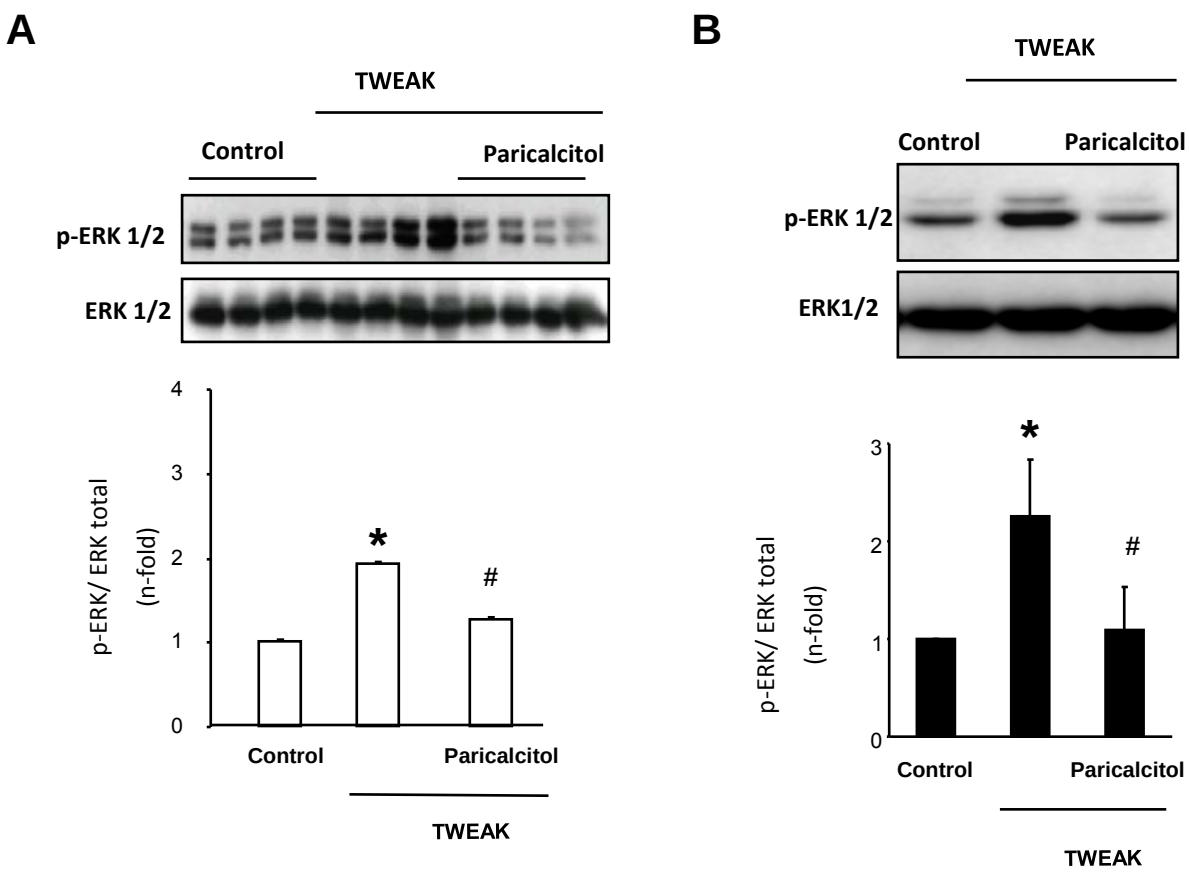

Figure 6. Paricalcitol inhibits TWEAK-induced activation of ERK signaling in the kidney and in tubular epithelial cells. By Western Blot, phosphorylated ERK (p-ERK) protein levels were evaluated in renal samples from mice (A) and cells (B) treated with TWEAK and/or paricalcitol. ERK 1/2 levels were used as loading controls. Data are expressed as mean ± SEM of 8-10 animals per group or 4 independent in vitro experiments. *p<0.05 vs control. # p<0.05 vs TWEAK.

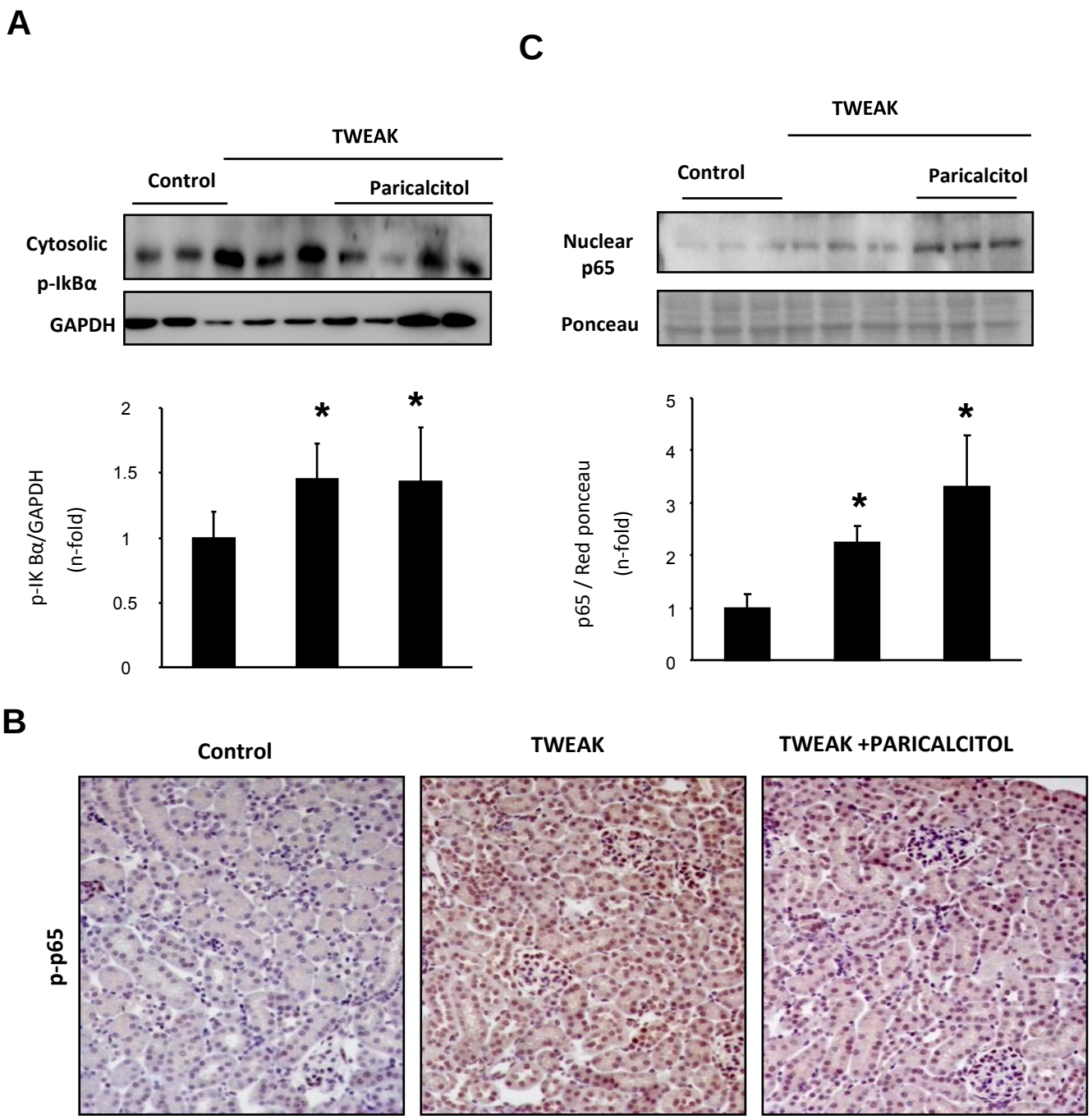

Figure 7. Paricalcitol did not modify TWEAK-induced activation of the canonical NF-κB pathway in the kidney. In renal samples from mice treated with TWEAK and or paricalcitol, cytosolic and nuclear proteins were isolated. Canonical NF-κB activation was assessed by cytosolic phosphorylated IκBα levels (A) and nuclear NF-κB p65 levels (C) by western blot. GAPDH and Ponceau red were used as loading control. Figure B shows the localization of active p65/relA (using an antibody against phosphorylated-p65) by immunohistochemistry of a representative mice from each group. In TWEAK-injected mice increased phosphorylated p65/relA (p-p65) immunostaining was found, mainly in the nucleus of tubular epithelial cells. Figures A and C show the quantification of the western blot expressed as mean ± SEM of 8-10 mice per group. *p<0.05 vs control.

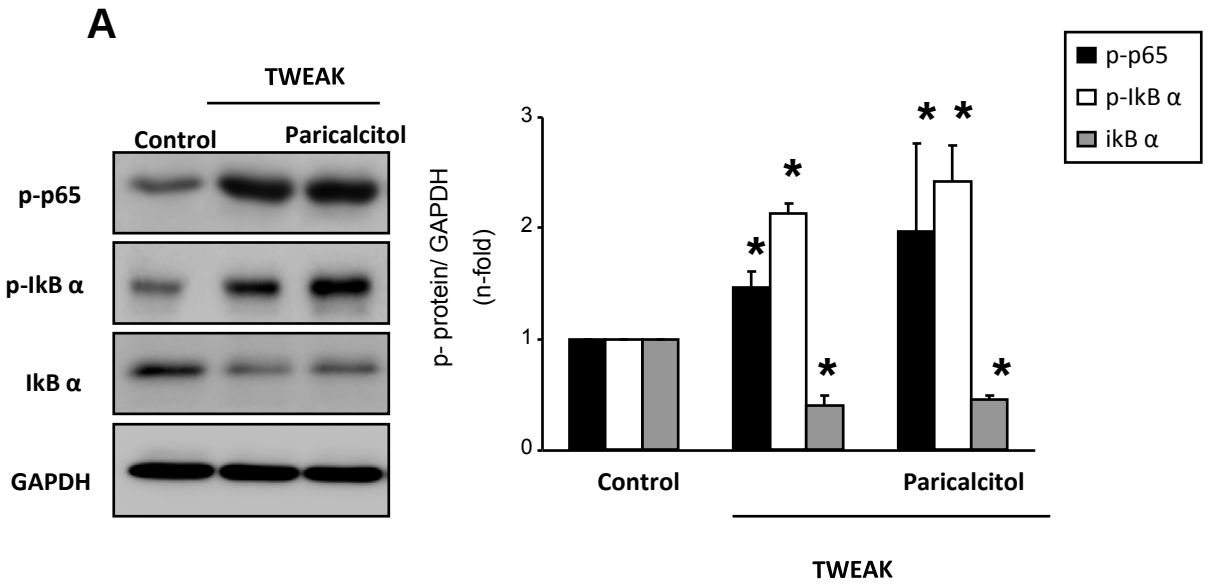

Figure 8. Paricalcitol did not modify TWEAK-induced activation of the canonical NF-κB pathway in tubular epithelial cells. Human tubular cells were pretreated with paricalcitol 48 hours previous to TWEAK stimulation (100 ng/ml) for 15 min. Activation of NF-κB1 was determined by evaluation of IκBα or p65 phosphorylated (A) in total protein extracts. Moreover, p65 levels were evaluated in cytosolic or nuclear fractions (B). Histone H1 were used as nuclear loading control. Figures show a representative experiment and data as mean ± SEM of 3-4 independent experiments. *p<0.05 vs control.

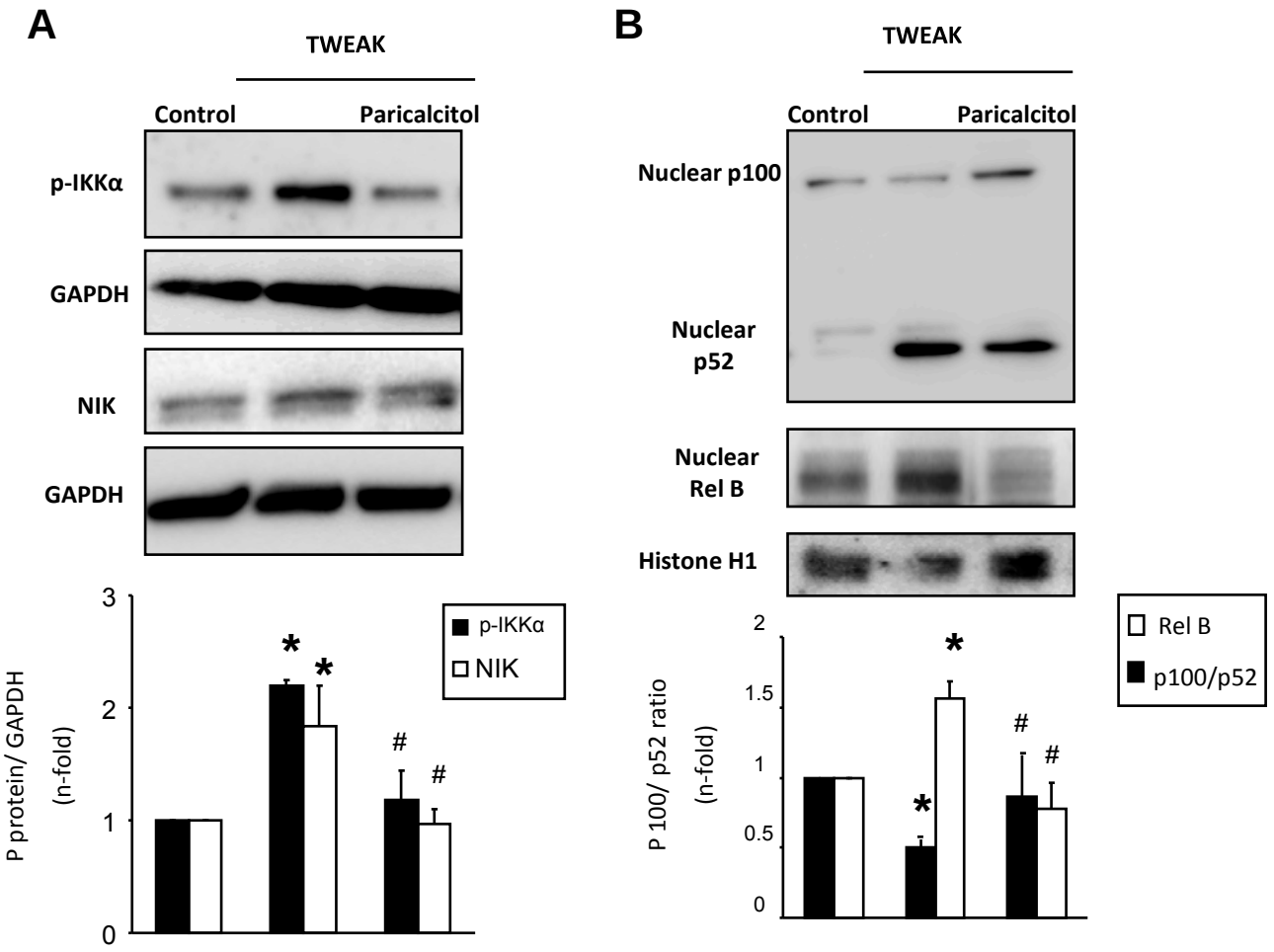

Figure 9. Paricalcitol prevents TWEAK-induced activation of the non-canonical NF-κB pathway in cultured tubular epithelial cells. Human tubular cells were pretreated with paricalcitol 48 hours previous to TWEAK stimulation (100 ng/ml) for 6 hours. Non-canonical NF-κB activation was assessed by IKK-α phosphorylation and NIK protein levels (A) in total protein extracts. Moreover, changes in nuclear NF-κB2 p100/p52 and RelB localization were also evaluated (B). Figures show a representative experiment and quantitation as mean ± SEM of 3-4 independent experiments. *p<0.05 vs control.

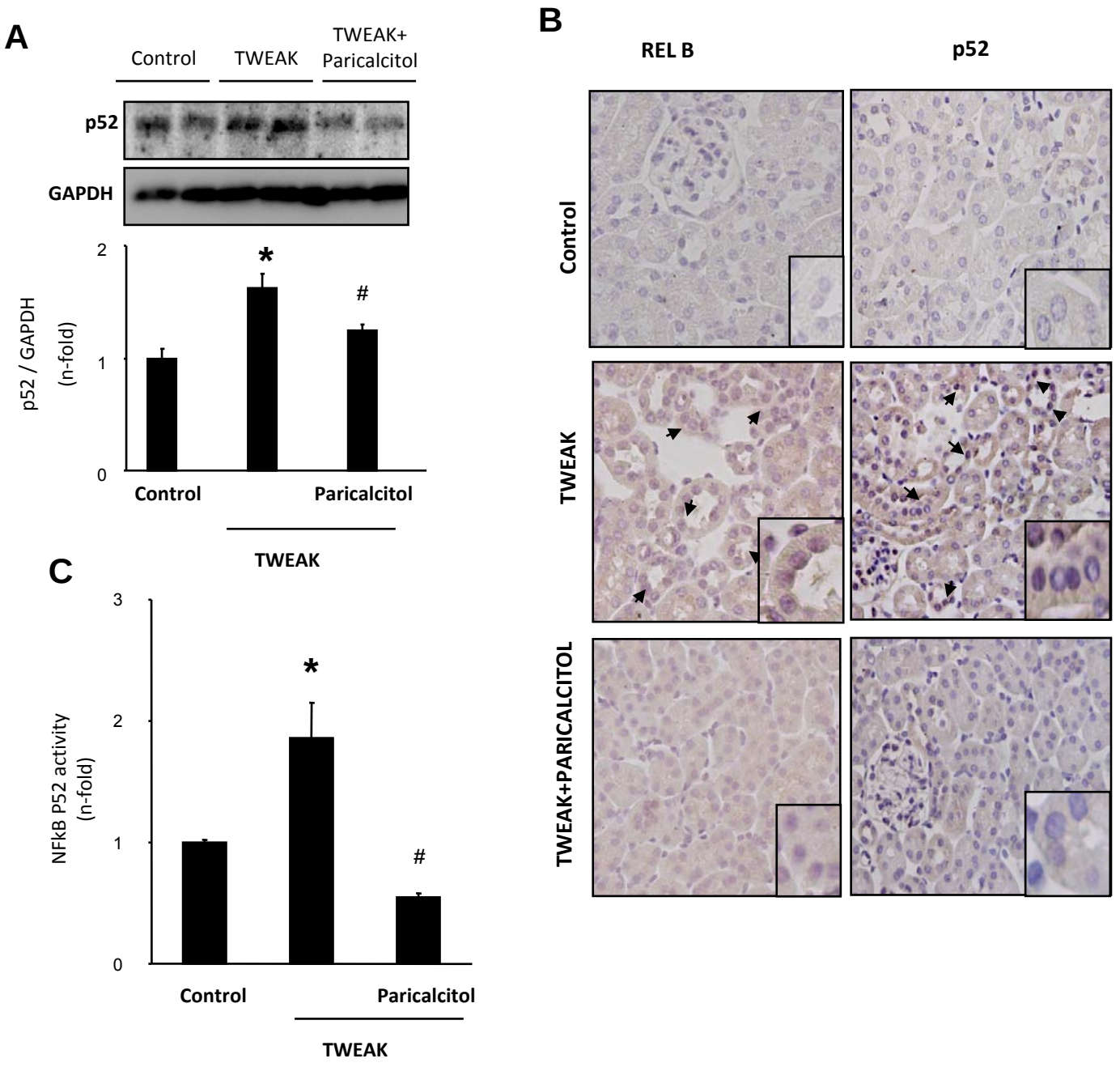

Figure 10. Paricalcitol prevents TWEAK-induced activation of the non-canonical NF-κB pathway in the kidney. (A) Non-canonical NF-κB activation was assessed by evaluation of renal levels of NF-κB2 p52 by western blot in kidney total protein extracts. (B) NF-κB2 p52 and RelB levels were studied by immunohistochemistry. In TWEAK-injected mice a clear nuclear immunostaining for both NF-κB2 p52 and RelB was found, that was prevented by paricalcitol. (C) Non-canonical NF-κB activation was assessed by ELISA in isolated renal nuclear proteins using an antibody that only recognizes NF-κB2 p52 when activated and bound to the NF-κB consensus site. Data are expressed as mean ± SEM of 6-8 animals per group. *p<0.05 vs control. # p<0.05 vs TWEAK.

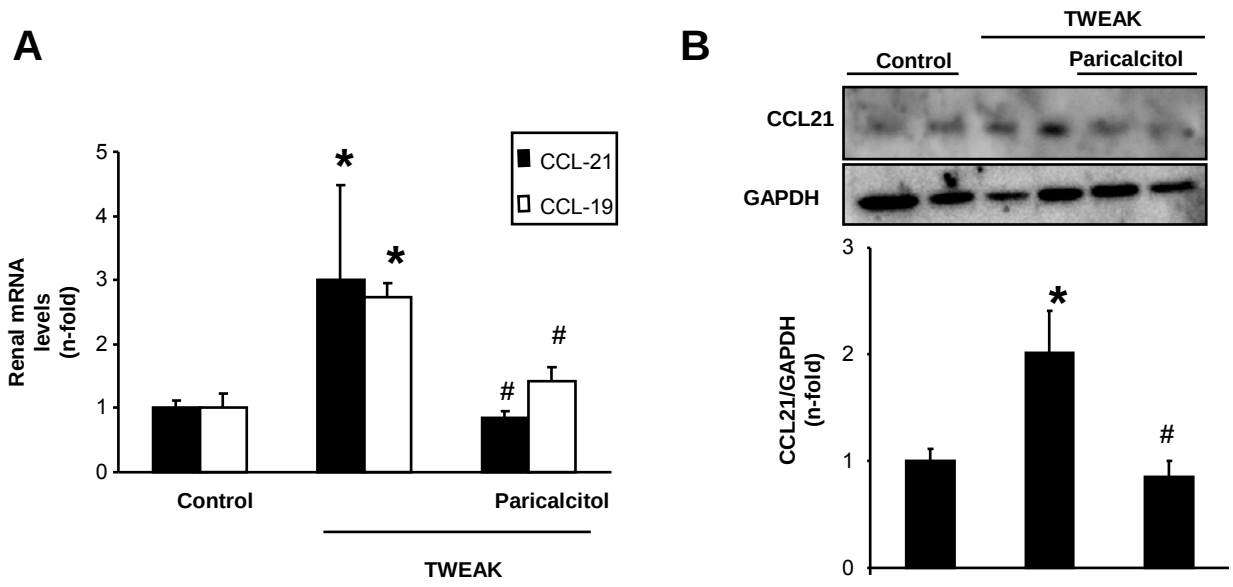

Figure 11. Paricalcitol inhibits TWEAK-induced upregulation of NF-κB2-regulated chemokines CCL21 and CCL19 the kidney. Gene expression was evaluated by real time PCR in renal samples from TWEAK-injected mice treated or not with paricalcitol. Figure A shows CCL21 and CCL19 gene expression levels as mean ± SEM of 8-10 mice per group. CCL21 protein levels were evaluated in total renal protein levels by western blot (B) or immunohistochemistry (C and D). (C) Localization of CCL21 showed increased immunostaining in tubular epithelial cells. (D) Quantification of CCL21 stained area was compared to total area, and expressed as mean ± SEM of 8-10 animals per group. $p<0.05$ vs control. # $p<0.05$ vs TWEAK. (E) Paricalcitol inhibits TWEAK-induced upregulation of CCL21 and CCL19 gene expression in cultured tubular epithelial cells. MCT cells were treated with 100 ng/ml TWEAK for 6 hours, some points were pretreated with paricalcitol. Gene expression levels were expressed as mean ± SEM of 4 independent experiments. *$p<0.05$ vs control. # $p<0.05$ vs TWEAK.

MATERIALS AND METHODS

Cultured cells

Human kidney proximal tubule epithelial cells (HK2 cell line, ATCC CRL-2190) were grown in RPMI 1640 with 10% fetal bovine serum (FBS), 1% glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin-transferrin-selenite and 36 ng/ml hydrocortisone in 5% CO_2 at 37° C. When cells reached 60 to 70% confluence, they were serum-depleted for 24 hours before the experiment.

Tubulo-epithelial proximal murine cells (MCT cell line) were originally obtained from Dr. Eric Neilson (Vanderbilt University), and used for gene expression studies. These cells were grown in RPMI 1640 with 10% FBS, 1% glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin in 5% CO_2 at 37°C. When cells reached 60 to 70% confluence, they were maintained in RPMI with 1% FBS for 24 hours.

Cells were stimulated with recombinant human soluble TWEAK (Millipore). In some experiments cells were preincubated for 48 hours with the VDRA paricalcitol (12 µmol/L, Abbott) prior to stimulation. DMSO, used as a solvent in some cases, had no effect on cell viability or on gene expression levels (not shown).

Protein Studies

The EGFR phosphorylation status was analyzed by Western blotting. Briefly, proteins were obtained using lysis buffer [50 mmol/L Tris-HCl, 150 mol/L NaCl; 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% Triton X- 100, 0.3% IGEPAL; 10 µl/mL protease inhibitor cocktail; 10 µl/ml PMSF and 10 µl/mL orthovanadate]. Protein content was quantified by the BCA method, using bovine serum albumin (BSA) as standard. Cell lysates (25 µg/lane) were separated on 6% to 12% SDS-polyacrylamide gels under reducing conditions. Samples were transferred to nitrocellulose membranes (Bio-Rad), blocked in 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Tween-20 and 5% milk, and incubated overnight at 4°C with the following antibodies [dilution]: anti-NF-κB2 p100/p52 [1:200], NF-κB p-p65 [1:200] and NF-κB p65 [1:200] (Cell Signalling); anti-phosphorylated EGFR on Tyrosine (Y) 1068 (p-$EGFR_{1068}$) [1:250] (Calbiochem), ADAM17 [1:1000] (Abcam), EGFR [1:250], p-ERK1/2 [1:200]; CCL21 [1:200]; p-IκBα [1:200] and IκBα [1:200] (Santa Cruz Biotechnology). Subsequently, membranes were incubated with a peroxidase-conjugated secondary antibody and developed using the ECL chemiluminescence kit (Amersham Pharmacia Biotech). Protein quality and transfer efficiency were assessed by Ponceau red staining (not shown). To assess protein loading membranes were labelled with anti-GAPDH [1:10000] (Chemicon International), p65 [1:200]; ERK2 [1:200] (Santa Cruz Biotechnology); α-tubulin [1:10000] (Sigma) or Histone H1 [1:1000]. Total proteins were used as control for phosphorylation studies. Films were scanned using the Gel Doc™ EZ machine imager and analyzed using the Image Lab 3.0 (Bio-Rad).

ELISA was used to quantitate the concentration of chemokines CCL2 and CCL5 (eBioscience). In renal samples, total protein content was determined by the BCA method, and equal amounts were analyzed. Data are expressed as fold increase over the mean of control levels.

ELISA-based NF-κB2 assay

Nuclear and cytoplasmic fractions were separated from renal tissues using the NE-PER Reagent (Pierce) following the manufacturer's instructions. In renal nuclear extracts, NF-κB2 DNA binding activity was measured by its binding to an oligonucleotide containing the NF-κB consensus site using a TransAM NF-κB Family kit (Active Motif) with an antibody that only recognizes active NF-κB2 p52.

Gene expression studies

Total RNA was isolated in Trizol (Invitrogen, Groningen, Netherlands) from tissues and cells. cDNA was synthesized using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using 2 µg of total RNA with random hexamer primers, following the manufacturer's instructions. We performed real-time PCR using probes (Taqman FAM. Fluorophore) from Applied Biosystems: VDR Mm_00437297_m1, ADAM-17 Mm_00456428_m1; CCL19 Mm_00839967_g1, CCL21 Mm_036466971_gH; CCR-2 Mm_00438270_m1; CCL-2 Mm00441242_m1; CCL-5 Mm_01302428_m1;IL-6 Mm_00446190_m1. Data were normalized to eukaryotic 18S ribosomal RNA 4210893E (VIC) and GAPDH Mm99999915_g1. The number of mRNA copies in each sample was calculated by the instrument software using the Ct value (shaped point arithmetic analysis on the thermocycler). The results were expressed as relative copy number compared to unstimulated cells or control kidneys, after normalization to 18S or GAPDH.

Design of the experimental model

All animal procedures were performed after approval by the Animal Ethics Committee of the IIS-FJD, following the guidelines of animal research in the European Community. Studies were done in female C57BL/6 mice (9-12 weeks, 20 g of weight), obtained from and maintained at IIS-FJD animal facilities in special pathogen-free conditions.

Mice received an intraperitoneal injection of 0.5 µg TWEAK/mouse (dissolved in saline) and were sacrificed 24 hours later. Controls were injected with the saline vehicle (n=8-10 mice per group). TWEAK endotoxin level were <0.1 ng/mg, confirmed by MALDI-TOF (not shown). Treatment with the VDRA paricalcitol (25 ug/kg/day, i.p., Abbot) was started 48 hours prior to TWEAK administration. Mice were sacrificed under anesthesia (ketamine and xylazine), perfused with cold saline and the kidneys were extracted. Then, kidney portions were fixed in formalin buffer for immunohistochemistry studies or immediately frozen in liquid nitrogen for gene and protein studies.

Renal histology and immunohistochemistry

Paraffin-embedded kidney sections (3 µm) were stained using conventional methods. Antigen retrieval was performed using the PTlink link system (Dako Diagnostics) with sodium citrate buffer (10 mmol/L) adjusted to pH 6-9 depending on the immunohistochemical marker, followed by immunohistochemical staining in a Dako Autostainer. Steps: 1) endogenous peroxidase blockade; 2)

primary antibody incubation; anti-CD3 (1:300; Dako) or anti-F4/80 (1:5000; Serotec); pEGFR1173 (1:200; Cell Signalling); NF-κB p-p65 (1:200; Cell Signalling); NF-κB2 p52 (1:50; Cell Signallling); RelB (1:50; Santa Cruz Biotechnologyes); CCL21 (1:90; Santa Cruz Biotechnologyes); 3) washing; 4) DUOFLEX Doublestain EnVision™ treatment, using 3,3'-diaminobenzidine as chromogen. For F4/80 staining a rabbit anti-rat linker was used before EnVision. Sections were counterstained with Carazzi´s hematoxylin. The intensity of the reactive mark was assessed using Image-Pro Plus software. For each sample (processed in duplicate in a blinded manner) the average value was obtained from the analysis of 4 fields (20X objective) as density/mm2 or percentage stained area vs total analyzed area. Data are expressed as n-fold increase over control mice, as mean ± SEM of 8-10 animals per group. Negative controls include: non-specific immunoglobulin and no primary antibody (not shown).

Statistical Analysis

The results shown in the text are expressed as mean ± SEM. The differences between groups treated with VDRA and controls were evaluated by Student`s t test and Mann-Whitney test, and $p < 0.05$ was considered significant. Statistical analysis was performed using SPSS statistical software (version 11.0, Chicago, IL).

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IV. DISCUSIÓN

1. CCN2 ES UN NUEVO LIGANDO DEL EGFR.

Mediante resonancia de plasmón superficial hemos demostrado la unión directa de CCN2 a la forma soluble del EGFR. CCN2 es una proteína modular, cuyos módulos pueden interaccionar con citoquinas, integrinas, proteínas de matriz extracelular, entre otras moléculas, regulando diversas funciones celulares. Así, se han descrito sitios de unión a IGF y TGF-β en el módulo N-terminal (Kim et al., 1997, Wang et al., 2001, Lam et al., 2003), y sitios de unión a integrinas en el C-terminal (Chen et al., 2004, Gao y Brigstock 2004). El dominio TSP-1 está implicado en la unión a proteínas de matriz extracelular, integrinas, proteoglicanos heparan-sulfato, proteínas relacionadas con el receptor de lipoproteínas de baja densidad y VEGF (Segarini et al., 2001; Gao y Brigstock 2004, Adams y Tucker 2000; Inoki et al., 2002; Leask y Abraham 2006). Los estudios de resonancia de plasmón superficial demostraron que el sitio de unión al EGFR se encuentra en el módulo C-terminal. Además, en células renales en cultivo, se observó que tanto la molécula completa CCN2 como el fragmento correspondiente al módulo C-terminal, denominado en esta tesis CCN2(IV), se unen al EGFR y activan su ruta de señalización. Hasta el momento se conocen 11 ligandos del EGFR entre los que se encuentran; EGF, TGF-α, HB-EGF, amfiregulina, betacelulina, epigen y epiregulina (Melenhorst et al., 2008). Los datos aquí mostrados amplían esta lista sugiriendo que CCN2 es un nuevo ligando del EGFR **(Figura 10).**

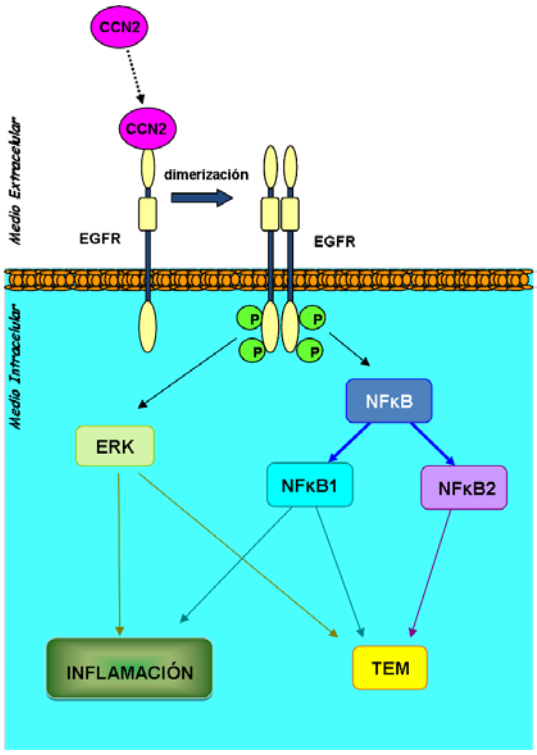

Figura 10: CCN2 es un nuevo ligando del EGFR: señalización intracelular y respuestas celulares.

El conjunto de experimentos *in vivo* e *in vitro* realizados en esta tesis sugieren que EGFR es un receptor funcional de CCN2 en el riñón. La administración sistémica en ratón de su fragmento C-terminal, CCN2(IV), mostró la activación de la ruta del EGFR, localizada principalmente en las células tubuloepiteliales renales, asociada a la inducción de una respuesta inflamatoria. Los estudios realizados utilizando el fragmento CCN2(IV) marcado con el fluoróforo Cy5 y el análisis por microscopia confocal permitieron localizar en el riñón *in vivo* los sitios de unión de esta proteína en las células tubuloepiteliales, tanto en el modelo de administración sistémica como en el de inyección renal directa

(ANEXO I, pag 160). Además, en estudios *in vitro* en este tipo celular se observó que CCN2(IV) interaccionaba rápidamente con la membrana celular permitiendo la formación del complejo CCN2-EGFR, induciendo la fosforilación del EGFR en sus tirosinas Y1068/Y1173 y desencadenando así la activación intracelular de esta vía de señalización.

Los ligandos de EGFR están presentes como precursores inactivos en la membrana celular y necesitan un procesamiento proteolítico, realizado principalmente por MMPs/ADAMs, para escindir su dominio extracelular y liberarse como ligandos maduros solubles que podrán activar el EGFR (por transactivación). En estudios *in vitro* se observó que ni la inhibición farmacológica de la actividad MMP, ni el silenciamiento génico específico de ADAM17, la ADAM más relevante en la patología renal, modularon la activación del EGFR inducida por CCN2(IV), confirmando la activación directa del EGFR por este nuevo ligando. La molécula completa de CCN2 puede ser digerida por diversas proteasas, dando lugar a varios fragmentos con actividad biológica, como el N-terminal o el C-terminal (De Winter et al., 2008, Hashimoto et al., 2002, Tam et al., 2009). En diferentes situaciones patológicas, incluida la ERC, la expresión renal de CCN2 está aumentada (Ito et al., 1998, Lau y Lam 1999, Blom et al., 2002, Gupta et al., 2000, Perbal 2004, Phanish et al., 2010). Se han observado niveles elevados de la molécula completa, y de los fragmentos N-terminal y C-terminal en la orina de pacientes con diversas nefropatías, incluida la nefropatía diabética, y se ha sugerido que CCN2 podría ser considerado como un biomarcador de daño renal (Riser et al., 2003). Sin embargo, hasta este momento no existen datos concluyentes sobre el proceso de proteólisis que da lugar a los distintos fragmentos de CCN2 en el riñón ni la potencial implicación de las ADAMs en este proceso, por ello serán necesarios estudios futuros para profundizar en este tema.

1.1 Papel de las integrinas en el complejo CCN2/EGFR y en su señalización intracelular.

Estudios *in vitro* han demostrado que la unión de moléculas a CCN2, a través de sus distintos dominios, regula diversas funciones celulares (Chen et al., 2004, Gao y Brigstock 2005). El modulo C-terminal de CCN2 se une a integrinas regulando proliferación, fibrosis e inflamación (Leask y Abraham 2006, Liu et al., 2006, De Winter et al., 2008), por lo que se ha sugerido que las integrinas podrían ejercer como receptores funcionales de CCN2, en concreto en la enfermedad tumoral.

Las integrinas son receptores heterodiméricos de las moléculas de adhesión de la superficie celular y de las proteínas de la matriz extracelular, que se componen de dos subunidades, α y β. Cada combinación αβ tiene propiedades de señalización específicas (Juliano 2002, Hynes 2002, Giancotti y Ruoslahti 1999). Hasta la fecha se han identificado, 18 subunidades α y 8 subunidades β, que forma al menos 24 integrinas αβ diferentes (Humphries et al., 2006), la mayoría de los datos existentes describen la interacción entre CCN1 y determinadas integrinas para regular adhesión, migración y proliferación en fibroblastos, células endoteliales, células de músculo liso vascular o monocitos (Chen et al.,2001, Grzeszkiewicz et al., 2001, Leu et al., 2003a, Babic et al., 1999, Schober et al., 2002 , Grzeszkiewicz et al., 2002). En el caso de las integrinas implicadas en las respuestas de CCN2, estas son dependientes del tipo celular. En células hepáticas estrelladas la integrina αVβ3 interacciona con el módulo C -terminal de CCN2 regulando la adhesión (Gao y Brigstock 2004), pero en células pancreáticas es la integrina α5β1 la encargada de mediar en procesos como adhesión, migración, mitogénesis y fibrosis (Gao y Brigstock 2005). En células tubuloepiteliales las subunidades de integrina αV y β3 han sido detectadas (Roy-Chaudhury et al., 1997, Londono et al., 2004), y se ha sugerido que la integrina αVβ3 podía regular la TEM (Shi et al., 2006), por ello se analizó la implicación de estas integrinas en la activación del EGFR inducida por CCN2(IV). El bloqueo de la integrina αVβ3 mediante diferentes

abordajes (anticuerpos neutralizantes o silenciamiento génico), pero no el de la integrina α5β1, inhibió la fosforilación del EGFR inducida por CCN2(IV) en células tubuloepiteliales. Mediante *cross-linking* y estudios de inmunoprecipitación se evaluó el papel de la integrina αVβ3 en la formación e interacción del complejo CCN2-EGFR. En estos análisis se observó la presencia de complejos CCN2-β3 y CCN2-αV. Además, el silenciamiento génico del EGFR no modificó la unión de CCN2(IV) a las subunidades que componen estas integrinas. Todo ello demuestra claramente la unión directa entre CCN2(IV) y la integrina αVβ3 en células tubuloepiteliales renales.

La capacidad de las integrinas de cooperar con TRKs, incluido el EGFR, para transducir señales proliferativas, regular la supervivencia celular y la migración, ha sido descrita previamente (Miranti y Brugge 2002, Schwartz y Ginsberg 2002). Las integrinas son capaces de formar complejos con el EGFR en la membrana celular y desencadenar la fosforilación independiente de ligando de los residuos tirosina Y845, Y1068, Y1086, y Y1173 en la molécula de EGFR (Moro et al., 1998), y parecen ser necesarias para las respuestas celulares desencadenadas por la activación del EGFR (Cabodi y Ginsberg 2004). Los datos descritos en esta tesis muestran que la integrina αVβ3 se une a CCN2(IV) y participa de la transducción de señales intracelulares inducidas por EGFR, pero no es necesaria para la formación del complejo EGFR-CCN2(IV). A pesar de que diversos estudios han sugerido que las integrinas podrían ser receptores funcionales de CCN2, nuestros resultados demuestran que el receptor funcional de CCN2 es EGFR y que las integrinas, a través de su interacción con este receptor, fomentan la señalización intracelular del mismo.

1.2. *Crosstalk* EGFR/TRKA en respuesta a la estimulación del CCN2.

TrKA es un miembro de la familia de receptores de membrana TrK (TrKA, TrKB, TrKC). Estos receptores interaccionan con neurotrofinas y forman homo/heterodímeros con el receptor de baja afinidad de neurotrofina, p75NTR (Allen y Dawbarn 2006). En células mesangiales se ha propuesto que TrKA podría ser un receptor funcional para CCN2 (Wahab et al., 2005). CCN2 también activa TrKA en cardiomiocitos murinos, y, como se muestra en esta tesis, en células tubuloepiteliales (Wang et al., 2010). Estudios previos han descrito el papel de los receptores TrKs en procesos asociados a la fibrosis, como es la TEM. En células de cáncer de pulmón, el bloqueo del receptor TrKB revirtió cambios asociados a TEM en estas células (Ricci et al., 2013). Nuestros resultados muestran que en células tubuloepiteliales el bloqueo de TrKA (mediante un inhibidor farmacológico, K252a) revirtió la TEM inducida por CCN2(IV), caracterizada por su cambio morfológico de célula epitelial a tipo miofibroblasto y por disminución de marcadores epiteliales, como E-Cadherina.

Mediante silenciamiento génico e inhibición farmacológica de EGFR y de TrKA hemos demostrando un *crosstalk* entre EGFR/TrKA en respuesta a la estimulación con CCN2(IV). Además, en monocitos se ha descrito la existencia de este *crosstalk* entre en EGFR/TrkA en respuesta a la estimulación de GPCRs (El Zein et al., 2010), lo que confirma la interacción entre estos dos receptores. El análisis del perfil fosfoproteómico de TrKA y EGFR muestra una a considerable similitud en la señalización originada por estos RTKs (Bradshaw et al., 2013). La complejidad en la señalización de los RTKs y sus interacciones entre ellos, requieren estudios futuros mas exhaustivos.

1.3. CCN2 a través del EGFR regula la inflamación renal y la TEM.

Estudios previos de nuestro grupo han demostrado que CCN2(IV) *in vivo* induce una respuesta inflamatoria renal temprana (Sánchez-López et al., 2009b). La intervención de la ruta del EGFR en los estudios *in vivo* e *in vitro*

desarrollados en esta tesis demuestran que CCN2, vía el módulo C-terminal, se une al EGFR regulando la expresión génica de factores proinflamatorios, como quimioquimas, y el reclutamiento de células inflamatorias en el riñón. Cabe destacar que la administración sistémica de CCN2(IV) en ratón induce una respuesta inflamatoria persistente, caracterizada por una activación local de la respuesta inmune Th17 asociada al aumento de los niveles de MCP-1 y a la presencia de células inflamatorias en el intersticio renal. Sin embargo, la evaluación de genes profibróticos demostró una leve regulación transitoria al alza en los niveles de α-actina de musculo liso (marcador de fibroblastos activados) y de ciertos componentes de la matriz extracelular, como fibronectina y procolágeno tipo I, pero no se produjo fibrosis renal, evaluada por el análisis en los niveles de colágeno acumulado en el riñón (ANEXO I, pag 174). Por este motivo, no se realizaron estudios *in vivo* evaluando una posible fibrosis modulada por la inhibición de la ruta del EGFR en este modelo crónico.

Como se ha comentado anteriormente, varios estudios *in vitro* han demostrado que CCN2, a través de la unión de diversas moléculas a sus distintos dominios, regula procesos diferentes. En células renales la región N-terminal de CCN2 se une a IGF-1 y sinergiza en la producción de proteínas de matriz (Kim et al., 1997, Wang et al., 2001, Lam et al., 2003). En células de *Xenopus,* CCN2, por el dominio rico en cisteínas, se une directamente a TGF-β actuando como un cofactor que fomenta la unión de TGF-β a sus receptores y potencia la vía de señalización de SMAD (Abreu et al., 2002). Los resultados de los estudios *in vivo* realizados en esta tesis empleando CCN2(IV) demuestran que el módulo C-terminal es clave en la regulación de la respuesta inflamatoria renal.

En distintas patologías humanas y modelos experimentales caracterizados por fibrosis, incluyendo enfermedades renales, se ha descrito sobreexpresión de CCN2 (Brigstock 1999, Phanish et al., 2010). Estudios previos demuestran la capacidad de CCN2 de regular fibrosis en células en cultivo y de actuar como mediador profibrótico de TGF-β (Grotendorst 1997), y además ha sido identificado como uno de los factores capaces de inducir TEM (Liu et al., 2006). La activación de la ruta del EGFR puede regular TEM en respuesta a diferentes estímulos, y parece ser clave en procesos tumorales (Misra et al., 2012, Chen et al., 2012, Serrano et al. 2014). La TEM es un proceso que aparece en diferentes contextos fisiológicos y patológicos, y ha sido un foco de intenso estudio en la nefrología ya que este proceso se ha identificado como un mecanismo desencadenante de la fibrosis tubulointersticial. En esta tesis se describe que en células tubuloepiteliales el bloqueo de EGFR (mediante inhibidores farmacológicos de la quinasa del EGFR) revirtió la TEM inducida por CCN2(IV), sugiriendo que el bloqueo del EGFR podría inhibir la fibrosis renal en patologías asociadas a la sobreexpresión renal de CCN2.

1.4. La vía ERK es clave en la señalización de CCN2/EGFR.

Un resultado relevante de esta tesis es la demostración de que CCN2 activa la ruta del EGFR/ERK en riñón. Los datos obtenidos en los estudios en las células tubuloepiteliales *in vivo* e *in vitro* demuestran que CCN2 induce la fosforilación del EGFR en sus tirosinas Y1068 e Y1173, asociadas a la activación de la cascada de las MAPKs (Pourazar et al., 2008, Rojas et al., 1996). Consecuentemente, el bloqueo del EGFR mediante silenciamiento génico o inhibición farmacológica de la quinasa del EGFR con Erlotinib, inhibió la activación de la ERK inducida por CCN2(IV). CCN2 y su módulo C-terminal CCN2(IV) activan la cascada de señalización de las MAPKs en diferentes tipos celulares (Gao y Brigstock 2005, Wu et al., 2006, Wahab et al., 2005, Yosimichi et al., 2006), incluyendo células tubuloepiteliales murinas (Sánchez-López et al., 2009b), y promueven diferentes respuestas biológicas dependiendo del tipo celular donde actué. CCN2 induce la proliferación y diferenciación de condrocitos a través de la activación de las quinasas

ERK y p38 MAPK (Yosimichi et al., 2006). En fibroblastos humanos, la activación de ERK y JNK regulan la expresión de proteínas de matriz, como α-SMA y colágeno I (Hu et al., 2013), y la vía ERK induce una respuesta proliferativa en respuesta a TGF-β1 en miofibroblastos (Huang et al., 2005). En células mesangiales, ERK media fibrosis, migración, reorganización del citoesqueleto y producción de algunas citoquinas (Crean et al., 2002, Wahab et al., 2007, Furlong et al., 2007). En células tubuloepiteliales, CCN2(IV) vía ERK induce la expresión de ILK (Liu et al., 2007), y de quimioquinas, como MCP-1 (Sánchez-López et al., 2009b). Los resultados de esta tesis demuestran que el bloqueo de la ruta EGFR/ERK inhibe la expresión de marcadores proinflamatorios en el riñón, así como la presencia de células infiltrantes (macrófagos/linfocitos T) en el intersticio renal, demostrando que esta ruta es clave en la regulación de la respuesta inflamatoria renal.

La activación de las MAPKs (ERK, p38 y JNK) es crucial en la génesis de la TEM, pero es dependiente del tipo celular (Bhowmick et al., 2001, Grotegut et al., 2006, Santibanez, 2006). Bibliografía previa describe el bloqueo de la quinasa ERK con el inhibidor U0126 revertió la TEM inducida por la combinación de TGF-β e IL1-β en células mesoteliales (Strippoli et al., 2008). En células tubuloepiteliales se ha observado que el bloqueo farmacológico de ERK, revirtió la TEM inducida por Ang II (Rodrigues et al., 2008). En esta tesis se ha descrito que CCN2 activa la quinasa ERK a través de la vía de señalización del EGFR. El tratamiento con un inhibidor de ERK (U0126), inhibió la TEM causada por CCN2(IV), mostrando que la vía EGFR/ERK es un elemento fundamental en la TEM.

1.5. La vía NF-κB es clave en la señalización de CCN2/EGFR.

Investigaciones previas de nuestro grupo han demostrado como CCN2(IV) es capaz de activar la vía canónica de NF-κB y han destacado el papel clave de la misma en los efectos proinflamatorios renales inducidos por este factor (Sánchez-López et al., 2009b). En células tubuloepiteliales se observó como el bloqueo del EGFR, con un inhibidor farmacológico o mediante silenciamiento génico, inhibió la activación de NF-κB en respuesta a la estimulación con CCN2(IV). En cambio, el bloqueo de TrKA, mediante los mismos abordajes, no fue capaz de inhibir la activación de esta ruta. Estos datos confirman que CCN2(IV) al unirse al EGFR activa la ruta canónica de NF-κB, regulando procesos inflamatorios, extendiendo estudios previos (Sánchez-López et al., 2009b).

Un resultado novedoso ha sido la demostración de que CCN2(IV) activa la vía no canónica de NF-κB, al ser capaz de inducir componentes de esta vía como p100/p52 y RelB, y aumentar la fosforilación de IKKα, quinasa específica de la vía en células tubuloepiteliales en cultivo. Además, se observó que el bloqueo farmacológico del EGFR revirtió la activación de la vía no canónica de NF-κB inducida por CCN2(IV). Existen pocos estudios sobre la relevancia de la activación de esta ruta no canónica en situaciones de daño renal, hecho que requiere investigaciones futuras.

Diversos estudios han descrito que NF-κB es un factor de transcripción que modula proliferación, apoptosis y migración celular, además de estar sobreexpresado en diversos tipos de cáncer (Karin et al., 2002). Estudios mediante *microarrays* han identificado diversas moléculas asociadas a TEM y metástasis, que además son genes diana para NF-κB, entre las que destacan Snail y E-cadherina (Huber et al., 2004, Jechlinger et al., 2003). El papel clave de la ruta NF-κB en la regulación de la TEM está bien establecido en el área de investigación sobre el cáncer. En células epiteliales mamarias la inhibición de NF-κB mediante transfección génica de un superrepresor de IκBα revirtió los cambios en marcadores de TEM inducidos por TGF-β. Por otra parte, la activación de NF-κB mediante un mutante constitutivamente activo de IKKβ indujo TEM y generó un fenotipo mesenquimal incluso en ausencia de

TGF-β (Huber et al., 2004). En células tubuloepiteliales, se ha observado que el tratamiento con un inhibidor de la activación de la vía canónica de NF-κB, parthenolide, o de la no canónica, lactacistina, revirtió la TEM inducida por CCN2(IV). En conjunto estos estudios *in vitro* muestran que CCN2(IV) a través de la unión al EGFR, activa la vía canónica y no canónica de NF-κB, y regula procesos asociados a daño en la célula epitelial y a la fibrosis, como es la TEM.

En conjunto, estos estudios *in vivo* e *in vitro* sugieren que CCN2(IV) se une directamente al EGFR y activa su vía de señalización permitiendo la modulación de mecanismos intracelulares, como la activación de la ERK y de la ruta del NF-κB, y la modulación de respuestas celulares incluyendo la regulación de la respuesta inflamatoria renal y TEM.

2. TRANSACTIVACIÓN DEL EGFR Y SU IMPLICACIÓN EN EL DAÑO RENAL.

El proceso de transactivación del EGFR puede ser inducido por la interacción de otras moléculas con sus receptores específicos, como es el caso de Ang II, TNF-α o TGF-β (Hobbs et al., 2011, Ebi et al., 2010, Lautrette et al., 2005), desencadenando la liberación de los ligandos específicos del EGFR que fomentan la activación del receptor y su posterior señalización intracelular.

2.1. CCN2 media la transactivación del EGFR inducida por TGF-β

TGF-β es un factor de crecimiento que regula una gran cantidad de procesos biol ógicos tales como morfogénesis, desarrollo embrionario, cicatrización e inflamación. Las alteraciones de TGF-β o de los componentes de su vía de señalización pueden contribuir a una amplia gama de enfermedades entre las que se encuentran patologías renales y cardiovasculares, fibrosis, cáncer y enfermedades congénitas (Lan y Chun 2012, Massague 2012, Redondo et al., 2012, Ruiz-Ortega et al., 2007, Santibanez et al., 2011). TGF-β es considerado una de las principales citoquinas profibrogénicas en el riñón (Bottinger 2007; Zeisberg y Kalluri 2013) y es capaz de estimular directamente la transcripción de genes de MEC, estimular la expresión tisular del inhibidor de las MMPs e inhibir la producción de colagenasas, favoreciendo la acumulación de MEC así como la inducción de TEM (Massague 2012, Neilson 2005).

La relación entre TGF-β y la transactivación del EGFR ha sido descrita previamente (Lee et al., 2010, Murillo et al., 2005, Uchiyama-Tanaka et al., 2002, Murillo et al., 2007, Wang et al., 2008). En hepatocitos y células tumorales gástricas, TGF-β induce la transactivación del EGFR mediada por ADAM17 (Murillo et al., 2005, Murillo et al., 2007, Ebi et al., 2010). Estudios previos han demostrado que TGF-β induce la producción de CCN2 y que este factor es un mediador *downstream* de las respuestas profibróticas de TGF-β (Ruiz Ortega et al., 2007). Los estudios *in vitro* realizados en células tubuloepiteliales demostraron que el bloqueo de CCN2 endógeno, mediante silenciamiento génico, disminuyó la activación de EGFR inducida por TGF-β después de 24 horas de incubación. Estos datos sugieren que CCN2 es necesario para la activación del EGFR inducida por TGF-β a tiempo largos, ampliando el actual paradigma que reconoce a CCN2 como un mediador de las acciones de TGF-β, incluyendo la señalización del EGFR **(Figura 11).**

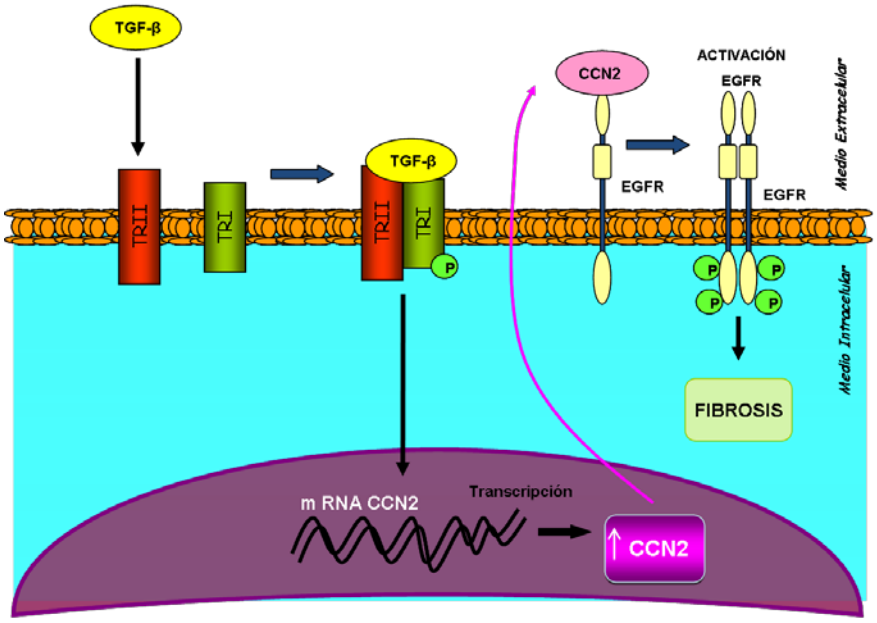

Figura 11: CCN2 es un mediador de las acciones de TGF-β, incluyendo la señalización del EGFR .

2.2. PTHrP y TWEAK nuevos factores capaces de inducir la transactivación del EGFR en el riñón

2.2.1 EGFR como vía implicada en las respuestas de PTHrP: EMT

La PTHrP es una proteína que se expresa en riñón (Clements et al., 2001) y tiene efectos proinflamatorios y moduladores del crecimiento en diferentes tipos celulares, incluyendo las células renales (Funk 2001, Ortega et al., 2006, Rámila et al., 2008). La PTHrP está sobreexpresada en diferentes nefropatías y esta sobreexpresión, se correlaciona con la aparición de proteinuria en modelos experimentales de nefropatía diabética o de daño tubulointersticial producido por sobrecarga proteica (Izquierdo et al., 2006, Largo et al., 1999). Estudios previos sugieren el efecto deletéreo inducido por la PTHrP a través de la vía de señalización del VEGF modulando la fibrosis, en el modelo de obstrucción unilateral del ureter (Ardura et al., 2008). Además, modelos experimentales de nefrotoxidad por acido fólico sugieren el rol clave de la PTHrP en la promoción de la fibrogénesis renal (Ortega et al., 2006). En esta tesis se demuestra como la PTHrP contribuye a la activación de la ruta del EGFR en el riñón. PTH1R, que pertenece a la familia de receptores GPCRs, se ha descrito como el receptor funcional de la PTHrP (Segre et al., 1993, Kolakowski et al., 1994). En células tubuloepiteliales en cultivo se observó que PTHrP(1-36) puede inducir la fosforilación de EGFR, con un patrón de respuesta similar al activado por otros ligandos de GPCRs. Los estudios realizados en ratones transgénicos que sobreexpresan PTHrP específicamente en el túbulo renal, han demostrado que tras un daño causado por la obstrucción unilateral del uréter, la activación de la vía del EGFR es mayor en riñones obstruido de los ratones que sobreexpresan PTHrP frente a los riñones dañados de los ratones de fenotipo salvaje. Se han descrito dos mecanismos de transactivación de EGFR inducidos por GPCRs (Fisher et al., 2003; Gschwind et al., 2001). Uno de ellos está mediado por segundos mensajeros, como Ca^{2+}, ROS y PKC, que activan a las MMPs/ADAMs lo que desencadena el procesamiento proteolítico de los ligandos de EGFR (Kodama et al., 2002; Soltoff 1998; Tsai et al., 1997, Ahmed et al.,2003; Syme et al., 2005; Eguchi et al., 2003). El otro mecanismo de transactivación es a través de la quinasa soluble Src que se fosforila y activa directamente al EGFR (Zhuang et al.,

2008). Los datos obtenidos sugieren que la transactivación de EGFR inducida por la PTHrP(1-36)/PTH1R en las células tubuloepiteliales se produce a través de ambos mecanismos, Src y MMPs, este último mediado por la PKC **(Figura 12).** Por esto, en un futuro sería interesante realizar estudios en profundidad sobre el papel de determinadas ADAMs, como ADAM17 en el proceso de transactivación del EGFR, así como los ligandos específicos responsables de la activación del EGFR inducida por PTHrP.

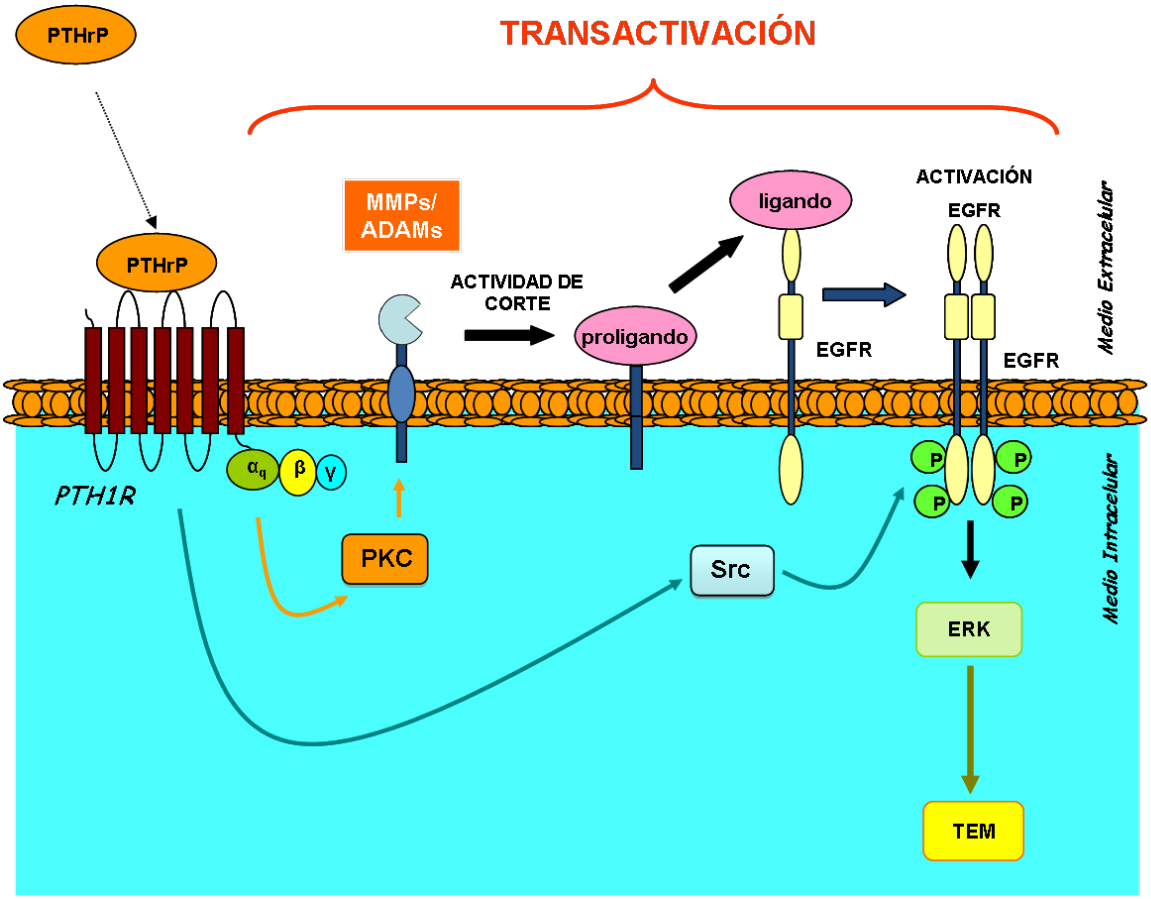

Figura 12: Respuestas celulares y señalización desencadenada por la transactivación del EGFR inducida por PTHrP.

En el modelo de daño renal en ratones transgénicos que sobreexpresan PTHrP, se observó que la activación de la ruta del EGFR estaba asociada a cambios en la expresión de marcadores de TEM, sugiriendo que la PTHrP modula la TEM en situaciones de daño renal. Mediante estudios *in vitro* se confirmó que la activación del EGFR juega un papel clave en la TEM inducida por PTHrP. En esta tesis se muestra como en las células tubuloepiteliales renales la PTHrP(1-36), vía transactivación del EGFR, indujo una serie de cambios fenotípicos relacionados con la TEM, incluidos la sobreexpresión y translocación nuclear de Snail, y la pérdida de marcadores como ZO-1 y E-cadherina, que son proteínas clave, en el mantenimiento de la polaridad basolateral y de las uniones intercelulares en el tubuloepitelio renal (Strutz y Müller 2006, Kalluri y Neilson 2003; Hinz et al 2001). Además, PTHrP(1-36) indujo un cambio fenotípico orientado hacia una morfología de tipo fibroblasto, asociado a inducción de marcadores mesenquimales, como α-SMA e ILK. En células renales, previamente se ha descrito el papel de Src como quinasa reguladora de la migración celular mediada por la fosforilación de ERK (Matsubayashi et al., 2004) y a su vez como proteína de importancia en TEM (Zhuang et al., 2005). También existe bibliografía previa acerca de otras quinasas como es el caso de la PKC, que participa del proceso de transactivación del EGFR mediado por MMPs (Ahmed et al., 2003, Syme et al., 2005, Yamashita et al.,2014) y que tiene también un papel relevante en TEM (Abera y Kazanietz 2015). Así, en

células tubuloepiteliales renales, se observó que la inhibición farmacológica tanto de Src (PP1), como de PKC (calfostina C), revirtió los cambios en TEM inducidos por PTHrP(1-36). Esta evidencia sugiere una asociación bidireccional entre Src y EGFR que conduce a su activación, necesaria para muchas funciones celulares, como la migración celular (Leu et al., 2003b) y como se describe aquí, en la TEM.

Como ya se ha comentado anteriormente la activación de las MAPKs tiene un papel clave en la TEM (Grotegut et al., 2006; Santibanez, 2006). En células tubuloepiteliales el bloqueo de la quinasa ERK inhibió la fosforilación del EGFR así como los cambios en TEM inducidos por la PTHrP. En células de túbulo proximal de conejos sometidos a estrés, la vía de ERK parece ser una consecuencia de la activación del EGFR inducida por PTHrP(1-36) asociada a su vez a la inducción de cambios en TEM (Zhuang et al., 2005). Esto sugiere que la activación de ERK parece ser un evento clave por el cual factores de crecimiento como la PTHrP y como se ha descrito antes CCN2, podrían cooperar para inducir TEM.

2.2.2. EGFR como vía de señalización clave en las respuestas de TWEAK: Papel en inflamación

TWEAK es una citoquina muy relevante en los procesos asociados al daño renal. Diversos estudios han demostrado que el bloqueo del eje TWEAK/Fn14 podría ser una nueva opción terapéutica para el daño renal (Ortiz A et al., 2009). Los datos experimentales presentados en esta tesis han demostrado que TWEAK activa la vía de señalización del EGFR en el riñón, concretamente en las células tubuloepiteliales. En estas células *in vivo* e *in vitro* TWEAK indujo la fosforilación del EGFR en sus tirosinas 1068 y 1173, así como la activación de la quinasa ERK y la expresión de factores proinflamatorios. Los estudios *in vitro* realizados demuestran que el silenciamiento génico de Fn14 inhibió la fosforilación de EGFR inducida por TWEAK. La inexistencia de una actividad tirosina quinasa intrínseca de Fn14, como en otros receptores de la familia TNFSR, indica que el EGFR tiene que activarse por transactivación. Múltiples estudios han mostrado el papel principal de las ADAMs en el proceso de transactivación del EGFR. Existen diversas disintegrinas de esta familia, pero es ADAM17 la más relevante en la patología renal (Melenhorst et al., 2008). El RNAm de ADAM17 se expresa constitutivamente en el riñón adulto y sus niveles se ven incrementados en situaciones patológicas (Melenhorst et al., 2009), como se observó en el estudio *in vivo* de administración sistémica de TWEAK. Los experimentos *in vitro* aquí realizados, mediante silenciamiento génico de ADAM17 e inhibición de los ligandos HB-EGF (CRM-197) y TGF-α (Anticuerpo neutralizante), mostraron que TWEAK tras su unión a Fn14, activa ADAM17 que libera los ligandos HB-EGF y TGF-α que a su vez inducen la activación del EGFR.

Diversos estudios experimentales han demostrado que TWEAK es un importante mediador del daño renal agudo mediante la modulación de la respuesta inflamatoria (Ortiz et al., 2009, Sanz et al., 2008). Algunos datos sugieren que ADAM17 podría estar implicada en inflamación (Ohtsu et al., 2006). En varios modelos animales de daño vascular, incluyendo hipertensión y ateroesclerosis, la modulación farmacológica y/o génica de ADAM17 o de ADAM10 mejoró la respuesta inflamatoria (Odenbach et al., 2011, Serino et al., 2007, Dreymueller et al., 2012). Bibliografía previa ha descrito que la interacción de TWEAK/Fn14 es capaz de inducir la activación de las MAPKs (JNK, p38 y ERK) (Donohue et al., 2003). En esta tesis se ha observado que el bloqueo de la vía EGFR/ADAM17, mediante el uso de inhibidores farmacológicos para EGFR (Erlotinib) o ADAM17 (WTACE2), previno la activación de la quinasa ERK y la inflamación renal inducida por TWEAK. *In vitro* el bloqueo de la quinasa ERK mediante inhibición farmacológica (U0126 y PD98059) revirtió la sobreexpresión de genes proinflamatorios inducidos por TWEAK, confirmando el rol del eje ADAM17/EGFR/ERK en la regulación de la expresión de factores proinflamatorios. Estos

datos sugieren que el bloqueo de la vía TWEAK/ADAM17/EGFR podría ser una nueva opción terapéutica para enfermedades renales inflamatorias **(Figura 13).**

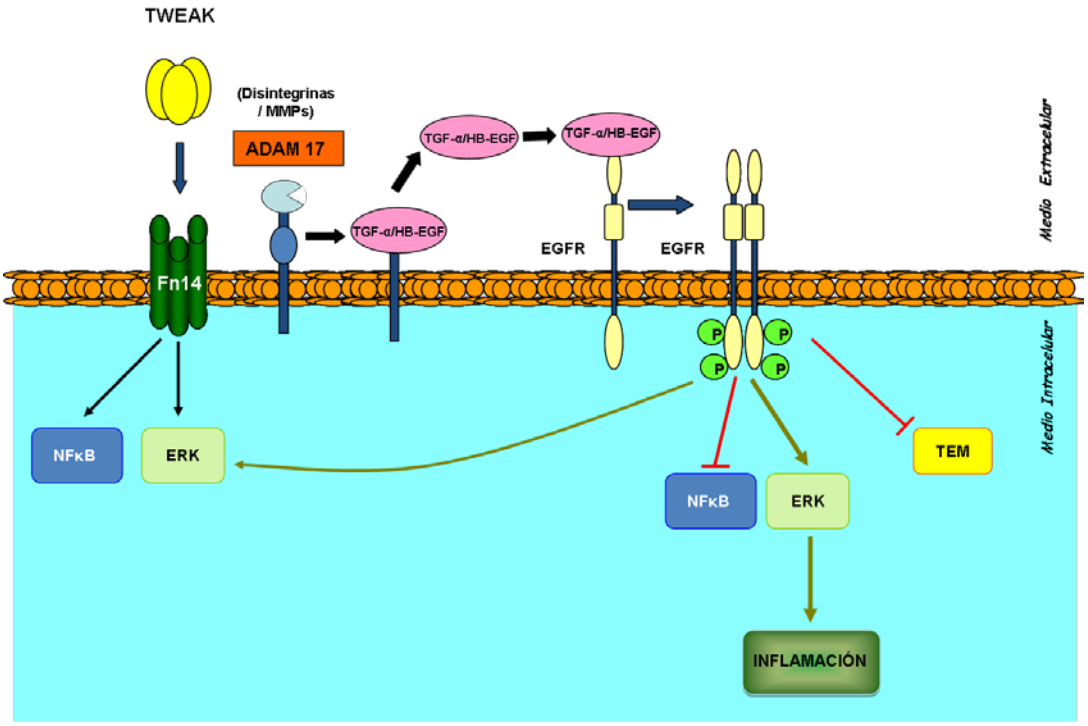

Figura 13: Respuesta celulares y señalización inducida por TWEAK a través de la transactivación del EGFR.

Diversos estudios han investigado el papel de los ligandos del EGFR en procesos de daño renal. Estudios realizados en ratones deficientes para HB-EGF, mostraron la implicación de este ligando en el daño podocitario en patologías como la glomérulonefritis progresiva. En estos estudios se observó que estos ratones deficientes para HB-EGF presentaban un menor infiltrado inflamatorio renal y unos niveles disminuidos de albuminuria previos a la aparición de una proliferación celular renal (Bollée et al., 2011). Diferentes estudios han demostrado que el HB-EGF regula la proliferación celular en queratinocitos humanos y en células epiteliales glomerulares (Hashimoto et al., 1994, Flamant et al., 2012). El ligando TGF-α se ha implicado en la fibrosis renal experimental inducida por Ang II (Lautrette et al., 2005). Dependiendo del ligando y del tipo celular, la activación del EGFR desencadena diferentes respuestas celulares. En estudios *in vitro* se ha observado que la estimulación con HB-EGF o TGF-α recombinante, aumentó la expresión génica de mediadores proinflamatorios, lo que sugiere que la activación del EGFR desencadenada por su interacción con ambos ligandos, podría estar implicada en la regulación de la respuesta inflamatoria renal.

TWEAK es una molécula que regula proliferación celular, diferenciación y apoptosis, dependiendo del tipo y el contexto celular (Chicheportiche et al., 1997, Sanz et al., 2011, Lynch et al., 1999, Perper et al., 2006). TWEAK induce apoptosis en líneas celulares tumorales y en monocitos (Burkly et al., 2007), mientras que en células tubulares renales incrementa su proliferación (Sanz et al., 2009). En astrocitos, la proliferación inducida por TWEAK a largo plazo, es regulada por la transactivación del EGFR vía TGF-α, así como por la activación de la quinasa ERK (Rousselet et al., 2012). Estudios utilizando ratones deficientes en TWEAK/Fn14 han demostrado que TWEAK participa en la regulación de la fibrosis renal (Ucero et al., 2013). TWEAK regula proliferación de fibroblastos renales,

pero no induce un aumento en la síntesis de colágeno en este tipo celular, por lo que los efectos profibróticos de TWEAK podrían ser atribuidos al aumento del número de fibroblastos renales. Otra posible explicación es por sus efectos sobre la TEM. En este sentido, la TEM inducida por TWEAK está mediada por la activación de la vía canónica de NF-κB, pero no por la transactivación del EGFR, como se observó mediante silenciamiento génico del EGFR (ANEXO I, pag 187). Diversos estudios experimentales muestran el papel de TWEAK en cáncer, y se están realizando ensayos clínicos explorando el rol de esta citoquina en procesos cancerosos mediante el uso de anticuerpos anti-Fn14 (http://clinicaltrials.gov/ct2/show/NCT00738764). Por otro lado, el silenciamiento génico de ADAM17 en células de carcinoma renal humanas, previno los cambios fenotípicos críticos asociados a la célula cancerosa, incluyendo el crecimiento del tumor, la inflamación y la invasión tisular (Franovic et al., 2006). Además, los inhibidores de la quinasa del EGFR también han demostrado efectos beneficiosos en el tratamiento del cáncer (Harada et al., 2011). Por todo ello, los resultados presentados en esta tesis acerca de cómo TWEAK induce la transactivación del EGFR vía ADAM17 podrían ser extrapolados a diferentes desordenes proliferativos.

En resumen, las consecuencias funcionales de la activación de la vía TWEAK/Fn14/ADAM17/EGFR en el riñón, incluyen la activación de vías de señalización *downstream* como la quinasa ERK, y la regulación de la respuesta inflamatoria renal, lo que sugiere que el bloqueo de la ruta de TWEAK/ADAM17/EGFR podría ser una nueva diana terapéutica para la inflamación renal **(Figura 13).**

3. EGFR COMO DIANA TERAPÉUTICA EN LA PATOLOGÍA RENAL

La señalización del EGFR presenta distintos roles en la fisiología renal humana modulando la hemodinámica glomerular y el metabolismo renal así como la homeostasis electrolítica renal (Melenhorst et al., 2008). Como ya se ha comentado en la introducción de esta tesis, el papel del EGFR en la patología renal es un poco contradictorio ya que se ha descrito que puede desempeñar acciones tanto beneficiosas como deletereas. En células tubulares proximales renales, ligandos como EGF (Zhuang et al., 2004), HB-EGF (Zhuang et al 2008), y epiregulina (Zhuang et al., 2007) pueden activar al EGFR e inducir proliferación regenerativa y migración. Estudios en un modelo de isquemia renal, demostraron que el HB-EGF induce la regeneración de células tubulares renales (Humes et al 1989) y protege a estas células de la apoptosis (Singh et al., 2007). En el modelo de obstrucción unilateral del uréter en rata, la administración de EGF, atenuó el daño renal y aceleró la regeneración tubular (Chevalier et al., 1999). Sin embargo, dependiendo de la localización, severidad, estímulo medioambiental y duración del proceso en el tiempo, la señalización del EGFR puede tener efectos muy distintos. Por ejemplo, en células tubulares proximales renales durante el proceso de regeneración post daño, necesitan proliferar y migrar, pero si estos procesos se alargan en el tiempo pueden desembocar en fibrosis y pérdida de funcionalidad (Melenhorst et al., 2008). Además las consecuencias de la señalización del EGF son dependientes de la especie, ya que en modelos de hidronefrosis la administración de EGF causa la muerte celular en ratones mientras que en ratas induce supervivencia (Kiley et al., 2005).

Diversos estudios experimentales han sugerido que el bloqueo del EGFR podría ser una importante herramienta para tratar la enfermedad renal (Melenhorst et al., 2008), sobretodo mediante la regulación de la proliferación celular y la fibrosis (Flamant et al., 2012). Los estudios realizados en el modelo de poliquistosis renal autosómica recesiva, mostraron que el tratamiento con un inhibidor de la quinasa del EGFR disminuyó la formación

de quistes y mejoró de la función renal. Además, empleando ratones mutantes WAVED 2 (que presentan una mutación puntual en el EGFR que reduce su actividad tirosina quinasa al 90%) se observaron resultados similares (Richards et al., 1998). En estudios posteriores en este mismo modelo se observó que el efecto beneficioso del bloqueo del EGFR se veía aumentado al combinarlo con la inhibición de ADAM17 (Sweeney et al., 2003). Estudios realizados en los modelos de masa renal reducida (nefrectomía subtotal) y de isquemia prolongada, mostraron que la expresión truncada de un dominante negativo para EGFR en células tubulares proximales disminuyó la infiltración de células mononucleares, la acumulación de colágeno intersticial y la proliferación tubular renal (Terzi et al., 2000). En ratas diabéticas, el tratamiento con inhibidores de la quinasa del EGFR disminuyó la proliferación de las células tubuloepiteliales, además de aumentar el tamaño glomerular (Wassef et al., 2004).

En modelos experimentales de hipertensión inducidos por varios factores (Ang II, leptina, monocrotalina, ET-1), el bloqueo del EGFR mediante diferentes abordajes; oligonucleótidos antisentido para EGFR, inhibidores de la quinasa del EGFR y ratones mutados WAVED-2, redujo los efectos característicos del daño tisular (Kagiyama et al., 2002, Jamroz-Wisniewska et al., 2008, Merklinger et al., 2005, Flamant et al., 2003). La administración sistémica de Ang II crónica induce lesiones fibróticas severas en el riñón. Sin embargo, la infusión de este péptido en ratones que expresan un dominante negativo del EGFR especifico de túbulo renal, los protegió frente a las lesiones producidas por Ang II. Además, se ha observado que en ratones *Knockout* para TGF-α y en ratones tratados con un inhibidor especifico de ADAM17, disminuye la fibrosis renal inducida por AngII (Lautrette et al., 2005).

4. IMPLICACIÓN DEL EGFR EN OTRAS PATOLOGÍAS

El origen del estudio de la vía de señalización del EGFR fue en la patología tumoral (Sibilia et al., 2007, Bronte et al., 2011). En diversos tipos de cáncer, incluidos los tumores de cabeza, cuello, pulmón, mama y tumores colorrectales, la familia de receptores ErbB (EGFR/HER1/ErbB1, HER2/neu/ErbB2, HER3/ErbB3 y HER4/ErbB4) se encuentra desregulada, es decir, estos receptores pueden encontrarse sobreexpresados produciendo un estimulación celular inapropiada (Sibilia et al., 2007, Martinez-Useros y Garcia-Foncillas 2015). En tumores malignos se ha descrito que HER2 y EGFR/HER1 están sobreexpresados, y se ha establecido que la sobreexpresión de EGFR se correlaciona con un peor pronóstico clínico (Mendelsohn y Baselga 2003). En el caso concreto de cáncer de pulmón se ha descrito una sobreexpresión del EGFR en un 90% de los tumores. Diversos mecanismos pueden desencadenar una expresión aberrante del EGFR, entre ellos destaca; la sobreexpresión de la proteína, su amplificación génica, la aparición de mutaciones, la sobreexpresión de ligandos del EGFR y la pérdida de los mecanismos reguladores de estos procesos (Martinez-Useros y Garcia-Foncillas 2015). Cabe destacar que las mutaciones en este receptor son uno de los indicadores que mejor se correlaciona con la eficacia de los inhibidores del EGFR (Martinez-Useros y Garcia-Foncillas 2015). En concreto, se ha observado un marcado efecto beneficioso y mayor respuesta al tratamiento con estos inhibidores del EGFR en pacientes que presentan mutaciones en el exón 19 (codones 746–750) y el exón 21 (sustitución de leucina por arginina en el codón 858 (L858R)) del EGFR con respecto a los pacientes que no presentan dichas mutaciones (Kosaka et al., 2011).

La vía de señalización del EGFR juega un papel crucial en procesos tumorales ya que modula el ciclo celular, inhibe la apoptosis, induce angiogénesis y promueve la motilidad de las células cancerosas y la metástasis (Herbst y Shin 2002). Los primeros abordajes terapéuticos contra EGFR comenzaron con el desarrollo de inhibidores farmacológicos reversibles del EGFR como Gefitinib (compite con ATP para unirse al dominio tirosina quinasa

intracelular del EGFR impidiendo su fosforilación). Posteriormente se diseñó el Erlotinib, que presentaba mejor perfil farmacocinético y de toxicidad (Li et al., 2007). Sin embargo, la respuesta a gefitinib y erlotinib no mejoró la supervivencia y solo se observaron mejoras en aquellos pacientes con mutaciones en el EGFR (Mok et al., 2009, Higgins et al., 2004). Otro inhibidor de reciente desarrollo es Afatinib que tiene especificidad dual por EGFR/ErbB1 y HER2/ErbB2 (Li et al., 2008). Debido a la implicación de estas vías en desarrollo embrionario y proliferación celular, la mayoría de los esfuerzos hasta ahora se centran en terapias anti-EGFR. Estas terapias conllevan el uso de inhibidores tirosina quinasa, que son pequeñas moléculas que se unen intracelularmente e interfieren con la señalización posterior del receptor, o el uso de anticuerpos monoclonales que bloquean el dominio extracelular de la quinasa del EGFR. Dentro de los anticuerpos desarrollados destaca Cetuximab que mejoró los índices de supervivencia en pacientes con cáncer de pulmón y cáncer colorrectal en combinación con la quimioterapia (Pirker et al., 2009, Jonker et al., 2007). En un estudio en cáncer renal, se observó que la administración de un anticuerpo quimérico murino/humano anti-EGFR (C225) inhibió el crecimiento de explantes de células de carcinoma renales normales en ratones NUDE (Prewett , 1998).

Se ha descrito el papel clave del EGFR en la homeostasis y en la patofisiología vascular, como se ha descrito en estudios con ratas espontáneamente hipertensas (SHR), cuyas células de músculo liso vascular presentan niveles elevados de EGFR y una mayor proliferación (Limas et al., 1980). También se ha observado su implicación en las complicaciones vasculares de la diabetes (Templeton et al., 2008; Costa et al., 2006; Veneri et al., 2005). En aterosclerosis se ha encontrado un incremento de la expresión del EGFR y de ciertos de sus ligandos como HB-EGF que parecen estar implicados en las distintas etapas del proceso aterogénico (Shafi et al., 2010; Gao et al., 2013, Stanic et al., 2012; Miyagawa et al., 1995). En modelos experimentales de hipertensión se ha observado que el uso de bloqueantes del EGFR, redujo la presión arterial y mejoró las lesiones vasculares (Griol-Charhbili et al., 2011; Benter et al., 2009; Flamant et al., 2003; Kagiyama et al., 2002; Ulu et al., 2010).

5. MODULACIÓN DE LA VÍA DEL EGFR EN LA PRÁCTICA CLÍNICA: MECANISMO POTENCIAL DEL EFECTO BENEFICIOSO DE LOS VDRAS.

La mayor parte de los pacientes con ERC presentan déficit de vitamina D activa (Valdivielso et al., 2009, Levin et al., 2006). Unos de los actuales tratamientos empleados en clínica en pacientes con ERC con demostrado efecto renoprotector son los VDRAs, en parte atribuidos a sus acciones antinflamatorias (Mizobuchi et al., 2007). Diversos estudios experimentales han reportado que la administración de vitamina D o de VDRAs mejoró la evolución de la enfermedad renal, disminuyendo la pérdida podocitaria, la proliferación celular y la fibrosis. En algunos de estos estudios se ha observado también una reducción de la presencia de células inflamatorias en el riñón (Mizobuchi et al., 2007, Kuhlmann et al., 2004. Panichi et al., 2001, Tan et al., 2006). Sin embargo, los mecanismos implicados en estos efectos antinflamatorios no están del todo esclarecidos.

En el estudio realizado en esta tesis en un modelo experimental de daño renal inducido por TWEAK, se observó que el VDRA paricalcitol inhibió la inflamación renal y la sobreexpresión de mediadores proinflamatorios, por un mecanismo dependiente de la inhibición de la vía de señalización de ADAM17/EGFR. Además, en células tubuloepiteliales en cultivo paricalcitol también inhibió la transactivación del EGFR y la posterior activación de la quinasa ERK inducida por TWEAK, sugiriendo que las acciones antinflamatorias de paricalcitol podrían ser debidas a la modulación de la vía de señalización del EGFR.

Diversos datos han mostrado la relación entre la Vitamina D y la vía de señalización del EGFR. Los primeros estudios se centraron en la unión del EGFR y la regulación de su expresión génica (Petkovich et al., 1987; Garach-Jehoshua et al., 1999, Tong et al., 1999, Koga et al., 1988). Posteriormente, se ha demostrado que la vitamina D y sus análogos aumentan la proliferación celular vía EGFR en diferentes tipos celulares a través de un mecanismo que incluye cambios en el tráfico del EGFR en la membrana y la regulación negativa del crecimiento celular inducido por EGFR (Valdivielso et al., 2009,González et al., 2002). Sin embargo, estudios recientes sugieren que estos efectos antiproliferativos podrían estar mediados por la modulación de la vía autocrina del TGF-α/EGFR (Cordero et al., 2002). Estudios previos han descrito que la vitamina D en monocitos/macrófagos es capaz de inhibir la producción de citoquinas inducida por LPS, a través de la activación de la MAPK fosfatasa 1, que inactiva p38 y JNK (Zhang et al., 2012). Nuestros resultados *in vivo* e *in vitro* identifican la vía de señalización del EGFR/ADAM17/ERK como una nueva diana de las acciones antinflamatorias de paricalcitol.

La vía de señalización clásica (o canónica) de NF-κB es uno de los principales mecanismos en la regulación de la inflamación renal. El bloqueo de la activación de la vía canónica NF-κB1 utilizando parthenolide, compuesto que inhibe la fosforilación de la subunidad inhibitoria IκBα y posterior activación del complejo p50/p65 NF-κB1, ha demostrado inhibir la respuesta inflamatoria renal inducida por TWEAK, al bloquear la regulación de diversos mediadores proinflamatorios como MCP-1 y RANTES (Sanz et al., 2008). Como se ha comentado anteriormente en el modelo de inyección de TWEAK la inhibición de la transactivación no moduló la vía canónica de activación de NF-κB1. En línea con estos resultados, el tratamiento con paricalcitol en este modelo de daño renal mediado TWEAK tampoco moduló la vía canónica de NF-κB1. Estudios realizados en células en cultivo han sugerido que la vitamina D es capaz de inhibir la vía canónica NF-κB1, determinado por cambios en la fosforilación de la subunidad inhibitoria IκBα y en la traslocación nuclear de la subunidad p65, como se ha descrito en estudios en células mesangiales, fibroblastos embrionarios murinos y células hepáticas estrelladas (Giarratana et al., 2004, Zhang et al., 2007; Sun et al., 2006). Sin embargo, los estudios *in vivo* realizados en un modelo de obstrucción unilateral del uréter mostraron que paricalcitol no fue capaz de reducir la activación de la vía canónica NF-κB1 (Tan et al., 2008), como hemos descrito aquí en el modelo de inyección de TWEAK. Cabe destacar que en el modelo de obstrucción, al igual que lo observado en respuesta al daño renal por TWEAK, paricalcitol inhibió la expresión de diversos genes proinflamatorios bajo el control transcripcional de NF-κB, como MCP-1 y RANTES. Mediante estudios de actividad de promotor se ha demostrado que paricalcitol es capaz de inhibir la unión de p65/Rel A al promotor de RANTES, pero no al de MCP-1. El mecanismo implicado en este proceso esta mediado por la formación del complejo VDR/p65, que bloquea la capacidad de p65 de transactivar la trascripción génica de RANTES (Tan et al., 2008). Estudios previos han demostrado que estos genes proinflamatorios pueden ser inhibidos mediante la modulación farmacológica de la activación de ERK (Rayego-Mateos et al., 2013b). Nuestros resultados muestran un mecanismo adicional de paricalcitol sobre el control de genes proinflamatorios, al actuar inhibiendo la ruta de señalización de EGFR/ERK, sin modular la vía canónica de NF-κB1, bloqueando la expresión de diversos genes proinflamatorios, como MCP-1 y RANTES.

Existen pocos datos acerca del papel de la vía no canónica de NF-κB2 en la enfermedad renal. TWEAK es una de las pocas citoquinas que es capaz de activar ambas vías de señalización de NF-κB, canónica y no canónica (Poveda et al., 2014). TWEAK en su activación de la vía no canónica NF-κB2 requiere la participación de la quinasa NIK, el procesamiento de la subunidad p100 y la expresión de Rel B, permitiendo la síntesis de citoquinas específicas de la vía, como CCL-21 y CCL-19 (Sanz et al., 2010a). La activación de esta ruta NF-κB2 se ha descrito en algunas

situaciones de daño renal, como diabetes experimental por estreptozotozina (Starkey et al., 2006) y nefrotoxicidad por ácido fólico (Sanz et al., 2010a). Se ha observado que el bloqueo de Rel B mediante un SiRNA específico, protegió contra los efectos dañinos producidos por la isquemia renal (Feng et al., 2009), pero existen pocos estudios del efecto de la inhibición de la vía de señalización NF-κB2 en enfermedades inflamatorias experimentales (Yang et al., 2010, Locke y Anderson 2011, Zhang et al., 2014, Cubillos-Zapata et al., 2014). Sorprendentemente, paricalcitol inhibió la activación de la vía NF-κB2 inducida por TWEAK *in vivo* e *in vitro.* En células tubulares en cultivo, paricalcitol inhibió la fosforilación de IKKα, y disminuyó los niveles proteicos de NIK, p52 y Rel B en respuesta a la estimulación por TWEAK. Además, paricalcitol redujo la acumulación de p52 y Rel B en el riñón de los ratones inyectados con TWEAK. La activación no canónica de NF-κB2 induce la transcripción de genes diferentes a los regulados por la vía canónica NF-κB1, entre los que se encuentran quimioquinas como CCL-21, CCL-19, CXCL13, CXCL12 y BAFF (Dejardin et al., 2002). Estudios efectuados en ratones *knockout* para p52/NF-κB2 demostraron que la activación de NF-κB2 es necesaria para su expresión (Carragher et al., 2004). Los estudios realizados en el modelo de TWEAK mostraron que paricalcitol reduce la expresión génica renal de CCL-21 y CCL-19, sugiriendo que las propiedades antinflamatorias los VDRAs podrían estar mediadas por la modulación de los genes controlados por NF-κB2.

En conclusión, los datos presentados en esta tesis muestran que los efectos antinflamatorios beneficiosos de los VDRAs como paricalcitol en la enfermedad renal, podrían explicarse por sus acciones sobre la ruta de ADAM17/EGFR/ERK y la vía no canónica NF-κB2 **(Figura 14).** Nuestros datos aportan información novedosa acerca de los mecanismos implicados en las acciones antinflamatorias previamente descritas de los VDRAs y podrían contribuir a un mejor diseño de futuros ensayos clínicos.

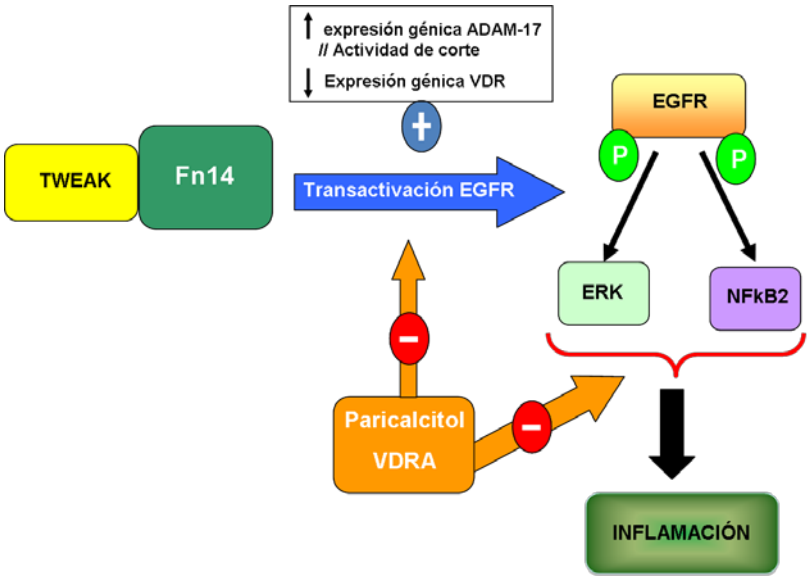

Figura 14: Efectos del Paricalcitol sobre la vía de señalización del EGFR inducida por TWEAK.

V. CONCLUSIONES

CONCLUSIONES

1. CCN2 es un nuevo ligando del EGFR. CCN2, a través de su módulo C-terminal, se une directamente al EGFR y activa diversas rutas de señalización intracelular, como la activación de la quinasa ERK y la ruta de NF-κB.
2. La administración sistémica de CCN2(IV) en ratón activa el EGFR en las células tubuloepiteliales regulando la inflamación renal.
3. En células tubuloepiteliales en cultivo CCN2(IV) induce TEM mediante la activación de la ruta del EGFR y de mecanismos de señalización intracelulares *downstream,* como ERK y NF-κB.
4. El bloqueo del CCN2 endógeno, inhibió la transactivación del EGFR inducida por TGF-β, confirmando y ampliando el paradigma de CCN2 como mediador de las acciones de TGF-β.
5. La sobreexpresión *in vivo* de PTHrP, específica en el túbulo epitelio renal, activa la ruta EGFR asociada a cambios en marcadores de TEM y daño renal. En células tubuloepiteliales en cultivo PTHrP induce TEM mediante la transactivación del EGFR por dos mecanismos: activación de MMPs (vía PKC) y activación de la quinasa Src.
6. TWEAK, mediante su interacción con su receptor Fn14, activa ADAM17 produciendo la liberación de los ligandos HB-EGF y TGF-α, lo que causa la transactivación del EGFR. El bloqueo de la ruta ADAM17/EGFR inhibe la inflamación renal inducida por TWEAK, sin modular la activación de la vía canónica de NF-κB.
7. El tratamiento con Paricalcitol, un análogo de la vitamina D, inhibe la respuesta inflamatoria renal causada por TWEAK en ratón, por un proceso mediado por la inhibición de la transactivación del EGFR y de la vía no canónica del NF-κB.

En resumen, estos resultados demuestran que la activación de la vía del EGFR, bien de forma directa por el nuevo ligando CCN2, o por transactivación causada por factores claves en el daño renal, como PTHrP y TWEAK, regula procesos inflamatorios y fibróticos. Estos datos sugieren que el EGFR es una potencial diana terapéutica para el tratamiento de la enfermedad renal crónica.

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VII. ANEXO I